Fate of *Escherichia coli* during Ensiling of Wheat and Corn†

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Received 17 November 2004/Accepted 23 March 2005

A recombinant *Escherichia coli* strain carrying a plasmid with an antibiotic resistance marker and expressing the green fluorescent protein was inoculated at a concentration of 3.8×10^8 CFU/g into direct-cut wheat (348 g of dry matter kg^{-1}), wilted wheat (450 g of dry matter kg^{-1}), and corn (375 g of dry matter kg^{-1}). The forages were ensiled in mini-silos. The treatments included control (no *E. coli* added), application of tagged *E. coli*, and delayed sealing of the inoculated wheat. Three silos per treatment were sampled on predetermined dates, and the numbers of *E. coli* were determined on Chromocult TBX medium with or without kanamycin. Colonies presumptively identified as *E. coli* were also tested for fluorescence activity. Addition of *E. coli* at the time of ensiling resulted in a more rapid decrease in the pH but had almost no effect on the chemical composition of the final silages or their aerobic stability. *E. coli* disappeared from the silages when the pH decreased below 5.0. It persisted longer in silages of wilted wheat, in which the pH declined more slowly. Control silages of all crops also contained bacteria, presumptively identified as *E. coli*, that were resistant to the antibiotic, which suggests that some epiphytic strains are naturally resistant to antibiotics.

Ensiling is a preservation technology for moist whole-plant forage crops which is based on lactic acid fermentation under anaerobic conditions, whereby lactic acid bacteria convert water-soluble carbohydrates into organic acids, mainly lactic acid. Fermentation results in a decrease in the pH and preservation of the moist forage. Many factors, including uncontrolled growth of microbial spoilers or pathogens, may affect the safety and quality of forage crops and silages. Good management practices and HACCP (hazard analysis and critical control points) should be applied to forage crops and feedstuffs as they are the first step in the human food production chain (14).

Among the factors considered hazardous in forage crops and silages are pathogenic enterobacteria, such as *Salmonella* and toxin-producing *Escherichia coli* (18). A slow decline in the silage pH favors the growth of enterobacteria, whereas rapid ensiling hastens elimination of these organisms (3, 11). Pathogenic strains of *E. coli* can cause severe illness in humans and animals, and the toxin-producing organism *E. coli* O157:H7 is of special concern; if conditions in silage are favorable for growth of this bacterium, it may cause intestinal disorders and mastitis in animals that consume the silage (13).

Cattle are a primary source of pathogenic *E. coli* O157:H7 (3), but this organism may be transmitted to crops and their products via shedding or through fertilization of fields with manure (15). Bach et al. (3) demonstrated that *E. coli* O157:H7 was eliminated from barley silage during a rapid ensiling fermentation, and Heron et al. (11) and Byrne et al. (7) obtained similar results for grass silage which was inoculated with *E. coli* O157:H7. However, it has been reported that *E. coli* O157:H7 might develop acid resistance through induction of an acid tolerance response and, consequently, would be able to survive at a pH as low as 3.4 (5, 12). It is known that *E.*

coli O157:H7 can survive in the human stomach and other extremely acidic environments (2, 6).

In Israel the hygiene of forage crops and their products (hay and silage) became an important issue because of intensive sewage irrigation. Weinberg et al. (17) showed that fresh forage crops which are irrigated by secondary-treated sewage water and well-preserved silages contain little or no *E. coli*. However, this species was detected in substantial numbers in decaying parts of silage (top and shoulders undergoing aerobic deterioration) and in feedstuffs in which the pH was above 5.0. It was hypothesized that the *E. coli* found in spoiled parts of silage and in various feedstuffs, such as total mixed rations, may have originated from the excreta of pigeons and other birds that live around dairy farms in Israel; alternatively, the *E. coli* may have developed from a dormant endogenous population in the silage following an increase in the pH.

The purposes of the present study were to monitor, under laboratory conditions, the fate of an *E. coli* strain that is resistant to antibiotics during ensiling of wheat and corn and to evaluate the conditions under which this organism might survive in silages.

MATERIALS AND METHODS

Ensiling. Two experiments were performed to study the fate of *E. coli* during ensiling. In the first experiment whole-plant wheat that had been harvested at the milk stage of maturity was used directly (D-wheat) or after wilting for 2 h on a warm day (W-wheat). In the second experiment we used whole-plant corn at the three-quarter milk line stage (at which most of the kernel contains solid starch). The plants were chopped into 2- to 3-cm segments with a Wintersteiger chopper (Austria). The chopped crop was ensiled in 250-ml mini-silos (sealed glass jars; Weck, Germany), which were stored at the ambient temperature (25 to 28°C). The treatments included controls (no *E. coli* added) and inoculation with an *E. coli* strain at a concentration of 3.8×10^8 CFU/g of forage⁻¹; the jars were sealed immediately in both cases. There were 18 silos per treatment; three of the jars were opened on days 1, 3, 6, 14, and 31 after ensiling in the experiments with wheat, and three of the jars were opened on days 1, 3, 6, 10, and 45 after ensiling in the experiments with corn. Three sealed silos from each treatment, which were stored until the end of the experiment, were left open for additional 5 days at room temperature to simulate aerobic exposure of the silages. In addition, in the experiment with wheat six silos that contained inoculated D-wheat and six silos that contained inoculated W-wheat were sealed 24 h after filling, to simulate

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† Contribution 421/04-E from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, 2004 series.

delayed sealing of silage. One silo from each of these treatments was sampled on days 1, 3, 6, and 14, and two silos were sampled on day 31.

The silages were analyzed for pH, dry matter, and numbers of *E. coli*, and the final silages were also analyzed for lactic acid and volatile fermentation products.

Bacterial strain. Plasmid pWM1029 (9) was introduced into the *E. coli* ATCC 25922 strain by a standard electroporation technique. This plasmid expresses both a kanamycin resistance gene and the green fluorescent protein (GFP) gene. The recombinant strain was grown overnight at 37°C in tryptic soy broth. The sizes of populations were determined by plating serial dilutions of the bacteria on tryptic soy agar (TSA) (Becton, Dickinson and Co., France) plates supplemented with 50 µg ml⁻¹ kanamycin (Sigma, United States). When the bacteria were grown on TSA, GFP fluorescence was easily observed under long-wavelength UV light.

Inoculation. The bacteria were applied by spraying 150 ml of the *E. coli* suspension onto 2 kg of each of the fresh crops (D-wheat, W-wheat, and corn) spread over a 1- by 2-m area, followed by thorough manual mixing. The suspension contained 5×10^9 CFU ml⁻¹; thus, a theoretical inoculation rate of 3.8×10^8 CFU g of crop⁻¹ was obtained.

Analyses. The dry matter of the fresh crops was determined by oven drying at 105°C for 24 h. Twenty-gram portions of the fresh crops or silage samples were extracted with 180 ml of distilled water in plastic bags for 3 min in a Stomacher blender (Seward Medical, United Kingdom). The pH was determined with a Toledo pH meter (Mettler, Switzerland). Lactic acid was determined spectrophotometrically as described by Barker and Summerson (4). Volatile fermentation end products in the aqueous extracts were determined with a gas chromatograph equipped with a semicapillary FFAP column (Hewlett Packard, Germany) over a temperature range from 40 to 230°C.

Enumeration of *E. coli* in the silage samples. The fresh crops or silage samples were extracted with a saline solution in sterile bags (Seward Medical, United Kingdom) for 3 min in a Stomacher blender. The numbers of *E. coli* were determined by plating 10-fold serial dilutions in duplicate on Chromocult TBX agar (Merck, Germany) with and without kanamycin by using the pour plate double-layer technique. The plates were incubated first for 2 to 3 h at 30°C to enable resuscitation of injured bacteria and then for 24 h at 42°C. Blue-green colonies were presumptively identified as *E. coli*. In order to confirm that the antibiotic-resistant colonies carried plasmid pWM1029, all colonies that developed on the Chromocult TBX agar to which antibiotic had been added were transferred with sterile toothpicks to TSA plus kanamycin for fluorescence determination. Colonies that appeared fluorescent under a UV lamp were considered to be the original *E. coli* inoculant.

To test for the presence of injured *E. coli* that could not grow in the selective medium at 42°C, samples were also inoculated into Luria broth (LB) (Hy-Labs, Israel) enrichment medium supplemented with kanamycin. One-milliliter saline samples from the extraction bags were added to 1 ml of double-strength LB containing kanamycin and incubated overnight at 30°C. Clear LB cultures indicated that there was no growth of bacteria, while turbid cultures indicated that there was possible growth of *E. coli*. To test if the tagged *E. coli* strain was present in the enrichment broth, 100 µl of a turbid LB culture was plated on Chromocult TBX agar plus kanamycin and incubated for 24 h at 42°C. Suspected *E. coli* colonies were transferred to TSA plus kanamycin and tested for fluorescence.

Yeasts and molds in the saline extracts were enumerated on malt extract agar spread plates acidified to pH 4.0 with 10% lactic acid, using 10-fold serial dilutions.

The statistical analysis included analysis of variance and Duncan's multiple-range test using the GLM procedure of SAS (SAS Institute, Inc., Cary, NC). The analysis was performed for each experiment separately. The experimental design was randomized, and the experimental units were the individual mini-silos. The statistical model tested the effects of the various crops and *E. coli* treatments on the dependent variables that were measured (pH, fermentation products, and log₁₀ *E. coli* counts). For the pH and *E. coli* counts the analysis was applied both by day and for the entire duration of the experiments. The root mean square of error was used as an estimate of the standard error of the mean.

RESULTS

The changes in pH during the ensiling of wheat are shown in Fig. 1. The pH of the D-wheat decreased rapidly, and there were no significant differences between the control and the inoculated silages at any time. In the silages of the W-wheat the pH decreased slowly. The pH values of the W-wheat con-

trol silages were significantly higher ($P < 0.05$) than those of the inoculated W-wheat for most of the sampling dates, except for days 1 and 31. The pH values of the silages prepared from the wilted wheat were significantly higher ($P < 0.05$) than those of the ensiled D-wheat throughout the ensiling period.

Table 1 shows the results of the analyses of the fresh wheat and its final silages. In the D-wheat (348 g of dry matter kg⁻¹) the major fermentation product was lactic acid, but ethanol was also found at high levels. Within the D-wheat treatments there were no significant differences in the analyses of the silages. The lactic acid concentrations were significantly ($P < 0.05$) lower in the W-wheat (450 g of dry matter kg⁻¹) silages than in the D-wheat, which is consistent with the higher pH values of the former. For silage treatments of the W-wheat, those inoculated with *E. coli* and sealed later had less lactic acid and more acetic acid than the other treatments. This result might reflect the more prolonged activity of the added *E. coli* in the silages which were sealed only after 24 h.

Visual appraisal of the final wheat silages after aerobic exposure revealed the beginning of spoilage, characterized by visible molds, and there was no difference between treatments. At the end of the aerobic exposure, the pH of the W-wheat silages increased by about 0.5 U, whereas the pH of the D-wheat remained the same as that in the sealed final silages. The relatively high ethanol levels found in the wheat silages could indicate yeast activity, and indeed, the populations of yeasts and molds were very large in all treatments following aerobic exposure.

Figure 2 shows the changes in the log₁₀ numbers of presumptive *E. coli* during the ensiling of the control wheat, as determined in medium with and without kanamycin; the numbers reflect the epiphytic *E. coli* populations. The values increased to more than log₁₀ 4.0 CFU g⁻¹ during the initial stages of ensiling but then decreased to below the detectable level (<log₁₀ 1 CFU g⁻¹). The bacteria disappeared more rapidly from the D-wheat silages than from the W-wheat silages. Interestingly, the control D- and W-wheat silages also contained presumptive *E. coli* cells which were resistant to kanamycin up to a concentration of log₁₀ 4.0 CFU g⁻¹; the numbers of these organisms were higher in the W-wheat silages, and they survived longer in these silages. The numbers of antibiotic-resistant *E. coli* in the control silages of the W-wheat were similar to the numbers of epiphytic *E. coli* that developed in media without kanamycin. The larger error on day 13 reflected the fact that in the W-wheat silages only two and one of three replicates exhibited *E. coli* counts in media without and with kanamycin, respectively.

Figure 3 shows the variation of the log₁₀ numbers of presumptive *E. coli* during ensiling obtained from the inoculated wheat by using media with kanamycin. The numbers were much higher than those in the control silages (Fig. 2) and reached log₁₀ 8.5 CFU g⁻¹. These values agree with the computed inoculation rate. The numbers of *E. coli* decreased during ensiling, but the organisms persisted longer in the silages of the W-wheat, similar to the epiphytic bacteria (Fig. 2). The root mean square of error reflects the variability in counts within a treatment.

One of the final three samples of inoculated D-wheat silages (day 31) whose sealing was delayed and which had been inoculated with *E. coli* produced colonies in kanamycin-containing

Root Mean Square of Error (R.M.S.E):

Day	0	1	3	6	13	31	36
R.M.S.E	-	0.075	0.04	0.04	0.05	0.07	0.20

FIG. 1. pH change during ensiling of wheat with *E. coli*. D, direct cut; W, wilted; EC, *E. coli*; delayed, delayed sealing. The solid lines indicate data for D-wheat, and the dashed lines indicate data for W-wheat. The arrow indicates the time when aerobic exposure was started.

Chromocult TBX agar after enrichment in LB, which suggests the possibility that the inoculated strain persisted throughout the ensiling period. No colonies were obtained from any other treatment after enrichment.

The GFP was expressed by most (about 70%) of the colonies of kanamycin-resistant *E. coli* derived from the inoculated silages in media with kanamycin.

The variation in pH during the ensiling of corn is shown in Fig. 4. Although the dry matter content of the whole-crop corn

was adequate for ensiling (375 g kg^{-1}), the pH of the control silages decreased more slowly than expected, possibly because of a lack of adequate lactic acid bacteria; the pH of the silages inoculated with the tagged *E. coli* decreased significantly faster ($P < 0.05$) because of the acids produced by this microorganism during the initial stages of the ensiling fermentation. Indeed, on day 10 after ensiling, the inoculated silages had more lactic and acetic acids than the control silages (Table 2). The initial decrease in pH caused by *E. coli* might have enhanced

TABLE 1. Analysis of wheat and its final silages (day 31)^a

Treatment or parameter	Dry matter (g kg ⁻¹)	pH	Concn (g kg [dry wt] ⁻¹)			After silo opening		
			Lactic acid	Ethanol	Acetic acid	pH	Concn of yeasts (log ₁₀ CFU g ⁻¹)	Concn of molds (log ₁₀ CFU g ⁻¹)
Fresh D-wheat	348	6.4 A						
Fresh W-wheat	450	6.4 A						
Control D-wheat		3.9 D	37 A	23 A, B	12 B	4.0 B	8.8	5.0
D-wheat + <i>E. coli</i>		4.0 D	38 A	24 A, B	12 B	4.1 B	8.4	3.6
D-wheat + <i>E. coli</i> , delayed sealing		3.9 D	37 A	28 A	12 B	3.9 B	8.3	4.6
Control W-wheat		4.8 B	12 B	27 A	5 B	5.4 A	9.6	4.3
W-wheat + <i>E. coli</i>		4.7 B	12 B	10 B	7 B	5.1 A	9.8	4.3
W-wheat + <i>E. coli</i> , delayed sealing		4.2 C	5 A	20 A, B	23 A	4.3 B	8.1	2.6
Root mean square of error	19	0.07	2.8	7.4	3.7	0.2		

^a Within a column, means followed by different letters are significantly different ($P < 0.05$). The effect of wilting was significant only for lactic acid ($P < 0.0001$), and within wheat type (D-wheat or W-wheat) the effect of treatment (control, *E. coli*, or *E. coli* with delayed sealing) was significant only for acetic acid ($P < 0.007$). No interaction was significant at $P < 0.05$.

Root Mean Square of Error (R.M.S.E):

Day	0	1	3	6	13
R.M.S.E	-	0.60	0.76	0.36	1.70

FIG. 2. Numbers of *E. coli* from control wheat silages. Bacteria were plated on Chromocult TBX agar with and without kanamycin. D, direct cut; W, wilted; +kan, medium with kanamycin. The solid lines indicate data for D-wheat, and the dashed lines indicate data for W-wheat.

the development of lactic acid bacteria in the silages, resulting in more lactic acid production.

Table 2 shows the results of the chemical analysis of fresh corn and fresh corn silages. On day 10 after ensiling the mean lactic acid contents of the control and inoculated silages were significantly different ($P < 0.0026$), but there were no significant differences in any of the chemical variables between the control and *E. coli*-inoculated silages. Overall, the lactic acid concentrations were lower than those usually found in corn silages, which accounts for the slow decline in the pH. Ethanol was the other major fermentation product. After aerobic exposure, more yeasts were found in the *E. coli*-treated corn silages than in the control silages.

Figure 5 shows the variation in the $\log_{10}$ numbers of *E. coli* during the ensiling of corn. The numbers of *E. coli* in the control silages determined on media without antibiotic increased initially up to $\log_{10}$ 4.0 CFU g^{-1} and then decreased concomitant with the decrease in pH, and the organism disappeared between days 10 and 45 of ensiling. The $\log_{10}$ numbers of kanamycin-resistant *E. coli* reached 4.0 on the first day after ensiling, but the organism subsequently disappeared. The numbers of *E. coli* in inoculated silages that grew on media with and without kanamycin followed similar patterns and decreased parallel to the pH decrease until the organism disappeared between days 10 and 45. It was expected that the numbers of tagged *E. coli* would be similar on media with and without kanamycin, because this microorganism grows on these media regardless of the presence of antibiotics. For each

medium (with or without kanamycin), the differences between the control and inoculated silages in $\log_{10}$ numbers of *E. coli* were significant on days 1 to 10 ($P < 0.05$). The large value for the error on day 6 reflects the fact that *E. coli* counts were obtained for only one of three inoculated silage samples.

On days 0 to 3 after ensiling, 90 to 100% of the colonies from the inoculated silages were fluorescent on TSA plus antibiotic; presumptive *E. coli* colonies obtained on day 6 and after day 6 did not express GFP on this medium.

For both wheat and corn, the interactions of day $\times$ treatment were significant ($P < 0.001$) for pH and $\log_{10}$ numbers of *E. coli* on media with and without antibiotics, which indicates that there were inconsistent differences in magnitudes between treatments during the experimental period.

DISCUSSION

This study was initiated in order to assess the safety of forage crops and silages made from these crops in Israel, where fields are irrigated with secondary-treated sewage water. Both raw sewage water and secondary-treated effluent may be sources of various pathogenic microorganisms and toxic chemicals. The cows in Israel are high-yielding dairy cows; they are intensively managed and, therefore, are sensitive to any change in feed quality or hygiene.

A previous study (17) revealed that the sewage-irrigated forage crops in Israel contain no or few *E. coli* and *Salmonella*. However, substantial numbers of *E. coli* have been found in

Root Mean Square of Error (R.M.S.E):

Day	0	1	3	6	13
R.M.S.E	-	0.57	0.60	0.18	2.07

FIG. 3. Numbers of *E. coli* from inoculated wheat silages. Bacteria were plated on Chromocult TBX agar with and without kanamycin. D, direct cut; W, wilted; EC, *E. coli*; delayed, delayed sealing. The solid lines indicate data for D-wheat, and the dashed lines indicate data for W-wheat.

samples taken from commercial silages in which the pH was between 6 and 7, and these silages were considered spoiled. Such samples were taken from the top layer or from the shoulders of the face, which are the parts of silages that are most susceptible to the air penetration that causes the pH to rise, with consequent spoilage. Such microorganisms in silage and in various feedstuffs may originate from the excreta of pigeons and other birds that live around dairy farms in Israel, or they may develop from regrowth of a small endogenous population after an increase in pH. The effect of these microbial populations on animal health is not known, but such microorganisms can cause illness in cattle (16) and may even be transmitted to humans (8, 15). In any case they serve as indicator microorganisms for the hygiene of food and feed commodities.

Usually, enterobacteria do not survive at a pH below 4.2 (10), such as the pHs that characterize well-prepared and managed whole-crop cereal silages. However, it has been reported that *E. coli* O157:H7 might develop acid resistance following induction of an acid tolerance response and, consequently, could survive at a pH as low as 3.4 (5, 12). Increased acid tolerance may enable pathogenic *E. coli* to survive in silage and in the stomach and cause intestinal disorders or mastitis in the animals fed this silage or even in humans (13).

In the current study an *E. coli* strain which is kanamycin resistant and expresses GFP was used. This strain is often used

as a quality control strain for antibiotic susceptibility studies. However, it has also been used for studying the survival of *E. coli* (for example, the survival on melons) (1). Up to 1 week after ensiling the numbers of *E. coli* from the inoculated silages were significantly ($P < 0.05$) higher than the numbers from the control silages (Fig. 2 and 3), and up to this stage this value served as an efficient tool for monitoring the survival of the exogenous *E. coli* during ensiling. After about 2 weeks the numbers of presumptive kanamycin-resistant *E. coli* in the control and inoculated silages were comparable. Therefore, the loss of fluorescence during the ensiling period may indicate loss of the tagged strain from the silage and a higher ratio of colonies of indigenous strains, including those which are kanamycin resistant. It is possible that epiphytic strains of *E. coli* are more resistant to the silage environment than the *E. coli* strain used in this study is. Therefore, additional research in which indigenous strains isolated from forage crops and silages are used is warranted.

The inoculation rate in the current experiments was very high (more than 10^8 CFU g⁻¹) in order to simulate a severe case of contamination. The results of these experiments indicated that the tagged *E. coli* usually disappeared from both wheat and corn silages after the initial stage of ensiling ended and the pH decreased to below 5.0. This finding is similar to the findings of other studies, in which grass and barley silages

Root Mean Square of Error (R.M.S.E):

Day	0	1	3	6	10	45	50
R.M.S.E	-	0.05	0.11	0.07	0.19	0.08	0.10

FIG. 4. pH change during ensiling of corn with *E. coli*. EC, inoculated with *E. coli*. The arrow indicates the time when aerobic exposure was started.

were used (3, 7). Furthermore, in the present study no *E. coli* was detected in the corn silages after the final silages were exposed to air, when the pH increased somewhat and conditions might have been more favorable for the growth of injured *E. coli*. This result may support the hypothesis that the *E. coli* in deteriorating parts of commercial silages might be introduced by an external source, such as birds' excreta and other contaminants. However, the fact that one sample of 31-day-old wheat silage, which was sealed 24 h after filling, supported growth of the tagged *E. coli* after enrichment suggests that *E.*

coli might survive in silage and grow when conditions are appropriate.

Addition of *E. coli* to silages accelerated the ensiling fermentation because of the faster acid production by this microorganism during the initial stages of ensiling. The rapid decrease in pH probably enhanced the development of lactic acid bacteria in the silage. In general, application of *E. coli* did not affect the composition of the final silages or their aerobic stability. The results of the current study are in agreement with those of Bach et al. (3), who found similar fermentation pro-

TABLE 2. Analysis of the final corn silages (day 45)^a

Treatment or parameter	Dry matter (g kg ⁻¹)	pH	Concn (g kg [dry wt] ⁻¹)			After silo opening		
			Lactic acid	Ethanol	Acetic acid	pH	Concn of yeasts (log ₁₀ CFU g ⁻¹)	Concn of molds (log ₁₀ CFU g ⁻¹)
Fresh corn	375	6.1						
Control (day 10)		4.8	6	2	2			
<i>E. coli</i> (day 10)		4.1	16	3	4			
Control (day 45)		4.2	18	17	3	4.4	5.0	NF ^b
<i>E. coli</i> (day 45)		4.1	21	11	5	4.2	8.7	NF
Root mean square of error	8	0.7	1.7	3.3	2.0	0.7		

^a The concentrations of yeasts in the final corn silages were log₁₀ 5.9 and 6.0 CFU g⁻¹ for the control and the *E. coli* treatment, respectively. On day 10 the mean lactic acid concentrations in the control and inoculated silages were significantly different ($P < 0.0026$).

^b NF, not found.

Root Mean Square of Error (R.M.S.E):

Day	0	1	3	6	10
R.M.S.E ¹	-	1.59	1.52	1.11	1.67
R.M.S.E ²		1.27	0.47	2.29	0.61

^{1,2} for counts on media without and with kanamycin, respectively.

FIG. 5. Numbers of *E. coli* from corn silages. Bacteria were plated on Chromocult TBX agar with and without kanamycin. C, control; EC, inoculated with *E. coli*; +kan, medium with kanamycin. The solid lines indicate data for control silages, and the dashed lines indicate data for inoculated silages. The arrow indicates the time when aerobic exposure was started.

files in control and *E. coli*-treated barley silages. We decided to add the delayed sealing treatments in order to prolong the favorable conditions for the tagged *E. coli* in the silage. In some respects, the delayed sealing can be viewed as additional replicates for the inoculated crops. However, these treatments resulted in lower pH values of the W-wheat compared with the corresponding inoculated silages which were sealed immediately (Fig. 1). This was probably due to much higher acetic acid production by the *E. coli* inoculant during the initial stage, when the silos were still open (Table 1), and in spite of the higher pK of acetic acid than of lactic acid (4.75 and 3.86, respectively).

Because the tagged *E. coli* strain may be more attenuated than the natural epiphytic bacteria under silage conditions, it may have disappeared from the silage more rapidly, and therefore, more experiments with field isolates are required before it can be concluded that adequate ensiling completely eradicates pathogenic *E. coli* strains.

An interesting result of the present study was the observation of numerous kanamycin-resistant *E. coli* strains in control silages. This finding probably reflects the widespread use of antibiotics in modern agricultural animal breeding practices,

which results in the acquisition of antibiotic resistance by the natural epiphytic flora. The results of our study indicate that among the natural epiphytic bacterial population there might be kanamycin-resistant bacteria which are more sensitive to low pH than the kanamycin-sensitive strains are. The prevalence and potential health risk of this population warrant more research.

Conclusions. Addition of *E. coli* at the time of ensiling resulted in a more rapid decrease in the pH but had almost no effect on the chemical composition of the final silages or their aerobic stability. *E. coli* disappeared from the silages when the pH decreased below 5.0. It persisted longer in silages of wilted wheat, in which the pH declined more slowly. Control silages of all crops also contained presumptive *E. coli* that was resistant to kanamycin, which suggests that some epiphytic strains are naturally resistant to antibiotics. Use of the tagged *E. coli* strain has been proved to be useful for monitoring the fate of *E. coli* in the initial stage of ensiling.

ACKNOWLEDGMENTS

We thank M. Brandl (Agricultural Research Service, Albany, Calif.) for providing plasmid pWM1029.

This work was supported by a grant from the Chief Scientist of the Israeli Ministry of Agriculture and Rural Development and by the Israeli Cattle Board.

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