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Efficient Improvement of Silage Additives by Using Genetic Algorithms

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The enormous variety of substances which may be added to forage in order to manipulate and improve the ensilage process presents an empirical, combinatorial optimization problem of great complexity. To investigate the utility of genetic algorithms for designing effective silage additive combinations, a series of small-scale proof of principle silage experiments were performed with fresh ryegrass. Having established that significant biochemical changes occur over an ensilage period as short as 2 days, we performed a series of experiments in which we used 50 silage additive combinations (prepared by using eight bacterial and other additives, each of which was added at six different levels, including zero [i.e., no additive]). The decrease in pH, the increase in lactate concentration, and the free amino acid concentration were measured after 2 days and used to calculate a "fitness" value that indicated the quality of the silage (compared to a control silage made without additives). This analysis also included a "cost" element to account for different total additive levels. In the initial experiment additive levels were selected randomly, but subsequently a genetic algorithm program was used to suggest new additive combinations based on the fitness values determined in the preceding experiments. The result was very efficient selection for silages in which large decreases in pH and high levels of lactate occurred along with low levels of free amino acids. During the series of five experiments, each of which comprised 50 treatments, there was a steady increase in the amount of lactate that accumulated; the best treatment combination was that used in the last experiment, which produced 4.6 times more lactate than the untreated silage. The additive combinations that were found to yield the highest fitness values in the final (fifth) experiment were assessed to determine a range of biochemical and microbiological quality parameters during full-term silage fermentation. We found that these combinations compared favorably both with uninoculated silage and with a commercial silage additive. The evolutionary computing methods described here are a convenient and efficient approach for designing silage additives.

The ensiling of forage crops in order to obtain winter or buffer feed for ruminant livestock is widely practiced in advanced management systems in temperate regions. The aim is to preserve crops having high moisture contents by encouraging rapid fermentation of water-soluble carbohydrates (WSC) in the crops to lactic acid by epiphytic lactic acid bacteria (LAB), which decreases the pH and inhibits the activities of plant enzymes and pathogenic or spoilage bacteria that could decrease the nutritive value of the silage.

Grass is the predominant crop ensiled in Europe, and 50 million tons of grass silage are made each year in the United Kingdom alone (23, 61, 62). As with maize, the main crop ensiled in the United States, high WSC levels and a low buffering capacity in this crop are conducive to rapid acidification by epiphytic LAB populations, and it is possible to make adequate silages without additives. However, under farm conditions the populations of epiphytic LAB are not always large enough or do not have a composition suitable for promoting efficient homolactic fermentation (16). Thus, efforts to obtain silage that has higher nutritional value and good storage properties have led to the development of a wide range of additives, most of which are sold as mixtures (4), that suppress or stimulate and direct what can otherwise be described as uncontrolled fermentation. However, most research on silage fermentation is of a strategic or applied nature, and thus we do not have a detailed understanding of the complex microbial and biochemical processes involved.

The additives that are used commercially include chemical inhibitors, such as acids, formaldehydes, and various salts, and biological stimulants (27, 65), including LAB and sometimes other bacteria that have specific antimicrobial properties (29, 31, 54). With organic or inorganic acids (formic acid or sulfuric acid), the preservative effect is due to a rapid decrease in the pH to a level at which only desirable microbes (mainly LAB) can survive. However, biological additives are becoming more popular in many countries for reasons of health and safety (63), as well as nutritional quality (10, 12).

The first generation of silage inoculants were selected strains of freeze-dried LAB that were added to supplement and compete with the epiphytic populations found on fresh herbage (44). These inoculants consisted of one or more strains of homofermentative (i.e., mainly lactate-producing) *Lactobacillus* spp. (predominantly *Lactobacillus plantarum*), often in combination with *Pediococcus*, *Enterococcus*, or *Lactococcus* spp. Although most silage inoculants are still freeze-dried and added as suspensions to forage, a new generation of freshly cultured inoculants (e.g., Live System) has been developed (30), in which LAB are cultured prior to application, which reduces the initial lag phase and improves silage fermentation characteristics. It has also been shown that freshly cultured inoculants consistently reduce proteolysis and increase the residual pro-

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tein content of silages (10, 12). Other additives also contain cell wall-degrading enzymes, such as cellulases and hemicellulases, which break down polysaccharides in order to ensure that there is an adequate supply of substrate for LAB and to enhance the digestibility of the silage (45, 46, 52).

Fermentation optimization is a field of study that has preoccupied many microbiologists (26, 53), and the approach used to design silage additives is an excellent example of the widely used and intuitive "educated guesswork" approach, in which a researcher uses selected levels of various additives based on past experience and knowledge of the system being studied. This approach is seldom completely rational, not only because of the sheer number of factors which may interact to determine the outcome of the fermentations but also because of the complex interactions between various parameters that may occur (28). For instance, in order to optimize a microbial growth medium containing 20 possible components at just two levels (present or absent), 2^{20} (ca. 1,000,000) possibilities must be explored. Thus, if there are even more ingredients (at a range of concentrations) or experimental conditions to be tested, it is apparent that only a fraction of the possible permutations can be tested.

Among the more rational search heuristics (decisions about which guesses to make) that have been used to optimize biological systems is the fractional factorial approach, in which carefully arranged subsets of all possible parameter combinations are tested sequentially (9, 37, 42). However, this strategy does not take into account epistatic and other interactions between parameters and requires assumptions to be made (e.g., that the response surface is unimodal), so that second-order polynomial equations may be applied. While using the fractional factorial and other related approaches is feasible when the number and range of parameters are limited, these approaches become logistically impossible in more complex situations (the number of possible permutations increases exponentially with the number of parameters or parameter levels being tested) (28).

Genetic algorithms (GAs), which were first devised by John Holland (21), are adaptive computer programs based on the principles of Darwinian selection and are the most commonly used evolutionary algorithms (5, 6, 18, 33, 43). It has been shown that empirical approaches like GAs, along with other methods, such as simulated annealing and neural networks (2, 19, 25, 38, 47, 55), can provide solutions for highly complex problems. GAs have provided acceptable solutions for a wide variety of combinatorial optimization problems, such as the classic "travelling salesman" problem or, more practically, the siting of retail outlets based on complex sets of geographic and demographic data (5, 18). In the microbiological context a key advantage of GAs over other methods of medium optimization is that no model is assumed.

The term "genetic" and the other evolutionary terms borrowed by Holland to describe the operation of GAs (see below) allude to similarities to the mechanisms of evolution and natural selection. In nature, a multidimensional fitness landscape (a term coined by the evolutionary biologist Sewall Wright [66]) is explored by a population of living organisms, and reproductive success is determined by individual fitness. This process is mediated by the generation of novel variation at genetic loci by mutation (which gives rise to allelic polymorphisms within a population) and the subsequent generation of novel gene combinations, usually via meiotic recombination. Once produced as described above, these gene combinations (genotypes) are subjected to the process of natural selection before the whole cycle is repeated many times in the process known as evolution. Populations of living organisms are thus able to optimize fitness by exploring multiple possibilities for solutions within the fitness landscape.

Traditionally, potentially useful LAB have been selected for use as silage additives by screening strains for desirable characteristics in laboratory media and then performing small-scale, full-term (3- to 4-month) ensilage experiments. These experiments are labor-intensive, and only a limited number of species or strains or combinations can be tested in each experiment. These facts led us to consider more logical and innovative approaches in order to simplify and improve selection procedures. The critical period of ensilage is the very early stage when plant enzymes and spoilage microorganisms are active at an almost neutral herbage pH (48). At this stage an adequate supply of available nutrients and very competitive LAB are needed to compete with the epiphytic microflora in order to reduce the pH rapidly. Because of this, we used the initial rates of lactate production, pH decline, and protein disappearance as criteria on which to base a novel short-term assessment of silage fermentation and quality. Here we describe this study, which was coupled with an innovative use of the GA approach in a series of proof of principle experiments performed to identify improved combinations of silage additive ingredients. The approach which we used was exceptionally successful.

MATERIALS AND METHODS

Source of herbage. Two adjacent plots of grass at the Institute of Grassland and Environmental Research, Aberystwyth, Wales, were used. The herbage in each plot was a mixture of perennial ryegrass (*Lolium perenne*) × Italian ryegrass (*Lolium multiflorum*) hybrid cv. Augusta, Italian ryegrass (*L. multiflorum*) cv. Abercomo, and perennial ryegrass (*L. perenne*) cv. Aberelan (all of which are widely used in the United Kingdom and parts of Europe). So that we could perform a series of experiments at fortnightly intervals and in order to minimize differences in herbage composition between experiments, the following cutting regime was adopted. One plot was cut on 19 May 1998, and the other plot was cut 2 weeks later. The herbage from these first cuts was discarded, and the five experiments were performed with grass that was harvested alternately from each half of a plot after successive 4-week periods of regrowth (i.e., second, third, and fourth cuts). The herbage was mown with a reciprocating mower (Agria 3000; Verkaufsgesellschaft GmbH, Moekmuehl, Germany), chopped into 3- to 4-cm-length pieces with an electrically operated forage harvester, and immediately transported to the laboratory, where it was mixed and separated into 100-g portions. Additives were applied in 2 ml of liquid as aerosol sprays with thorough mixing of the herbage. Buffering capacity was measured by the method of Playne and McDonald (36), while the total nitrogen content and WSC levels in the fresh herbage were measured as described by Merry et al. (32).

Additive treatments. Eight different ingredients were used as additives (Table 1) at six different levels, including zero (no addition). Dilutions of the freeze-dried and fresh inoculants were prepared by using distilled water in order to obtain the five levels used in the GA experiment. Additives A through G were mixed to obtain the appropriate concentrations for each treatment, and 1 ml was sprayed onto 100 g of herbage (Table 1). Additive H (fructose-glucose [60:40, wt/wt]) was applied separately after dilution with distilled water, and 1-ml portions were sprayed onto 100-g portions of herbage. Each treatment combination was assigned an index number which indicated the level of each additive (for example, a treatment with index no. 24432503 contained level 2 of additive A, level 4 of additive B, level 4 of additive C, etc., as shown in Table 1).

In order to calculate application rates for both freshly cultured and freeze-dried inoculants, viable counts (24) were determined by preparing appropriate 10-fold serial dilutions with 0.25× Ringer's solution (catalog no. BR52; Oxoid, Unipath Ltd., Basingstoke, United Kingdom) and using 1-ml aliquots of the appropriate dilutions to prepare pour plates containing MRS agar (catalog no. CM361; Oxoid). The plates were overlaid with a thin second layer of MRS agar and incubated at 30°C for 3 days. *L. plantarum* and *Pediococcus pentosaceus* (additives D and E) were cultured in MRS broth (catalog no. CM359; Oxoid) at 30°C for 24 h and then subcultured in MRS broth for 24 h before they were used; these cultures contained on average 2.6×10^9 and 5.3×10^9 CFU g^{-1}, respectively.

Preparation and analysis of silages. Samples were taken from the fresh herbage prior to ensilage and stored at −20°C before analysis. Immediately after treatment, 100-g portions of treated herbage were packed into glass tubes. The tubes were sealed with rubber bungs and air locks, incubated at 18°C, and destructively sampled after 2 days. Each sample was mixed well and then divided into portions which were used for chemical analyses, including dry matter (DM), lactic acid concentration, pH, and free amino acid level analyses.

TABLE 1. Silage additives[a]

Additive		Supplier	Concn[b]				
Designation	Organism or compound(s)		Level 1	Level 2	Level 3	Level 4	Level 5
A	Freeze-dried *Lactobacillus plantarum*	Biotal Ltd.	10^3	10^4	10^5	10^6	10^7
B	Freeze-dried *Pediococcus pentosaceus*	Biotal Ltd.	10^3	10^4	10^5	10^6	10^7
C	Freeze-dried *Lactobacillus buchneri*	Biotal Ltd.	10^3	10^4	10^5	10^6	10^7
D	Fresh *Lactobacillus plantarum*	Biotal Ltd.	10^3	10^4	10^5	10^6	10^7
E	Fresh *Pediococcus pentosaceus*	Biotal Ltd.	10^3	10^4	10^5	10^6	10^7
F	β-Glucanase	Quest International, Ashford, United Kingdom	1.50	3.75	7.20	14.40	43.20
G	Xylanase	Megazyme, Bray, Ireland	1.13	2.84	5.46	10.92	32.76
H	Fructose-glucose (60:40)	BDH, Poole, United Kingdom	0.2	1.0	2.0	5.0	10.0

[a] The eight additives were each added at one of six levels (including zero [no additive]). For each inoculant, level 3 represented the standard concentration recommended for silage inoculation by the supplier. The concentrations of the freeze-dried preparations of *L. plantarum*, *P. pentosaceus*, and *L. buchneri* supplied were 2.76×10^{11}, 4.46×10^{11}, and 3.99×10^{11} CFU g^{-1}, respectively. The fresh *L. plantarum* and *P. pentosaceus* preparations were obtained from 24-h (log-phase) cultures that had been inoculated with freeze-dried material (see above). The concentration of the β-Glucanase from a genetically modified *Bacillus* sp. and the concentration of the xylanase from *Trichoderma viride* were 15,000 and 11,375 IU ml^{-1}, respectively (as measured by Biotal Ltd.). Details of how additives were diluted and applied to the chopped grass are given in the text.

[b] The units for the *L. plantarum*, *P. pentosaceus*, and *L. buchneri* preparations were CFU per gram of fresh matter; the units for the β-glucanase and xylanase preparations were international units per gram of fresh matter; and the units for the fructose-glucose preparation were milligrams per gram of fresh matter.

The DM contents were determined by freeze-drying the herbage samples to a constant weight. Lactic acid concentrations and pH were measured as described by Merry et al. (32). A sample was prepared for free amino acid analysis by adding 80 ml of distilled water to 10 g of sample and placing the preparation in a stomacher (model 400 BA7021 lab blender; Seward Ltd., London, United Kingdom) for 10 min. The preparation was then filtered through Whatman no. 1 filter paper. An appropriate dilution was prepared with distilled water, and the concentration of free amino acids was determined by using the method described by Rosen (39) and ninhydrin, as adapted by Winters et al. (64).

Operation of GAs. In a GA experiment a population of individuals, each of which consists of a string of numbers (and each of which represents a potential solution to the problem being optimized), undergoes a process analogous to evolution in order to derive an optimal or nearly optimal solution. The parameters stored by each individual are used to assign to it a fitness value (defined as a single numerical value which indicates how well the solution obtained with that set of parameters performs), which in "traditional" GA scenarios is usually calculated by in silico modelling of the optimization problem. New individuals in the GA experiment (whose fitness is tested in each successive generation) are then generated from members of the current population by processes analogous to biological asexual and sexual reproduction. Asexual reproduction (or mutation) in a GA experiment is performed by randomly selecting a parent with a probability proportional to its fitness and then randomly changing one or more of the parameters which it encodes. Sexual reproduction (or crossover) is achieved by randomly selecting pairs of parents (at a rate related to the fitness of each parent) and generating two new individuals by recombining parameters between parents at one or more randomly selected crossover points. The processes of fitness evaluation and generation of new populations of individuals are repeated through successive cycles of the GA, and the overall fitness of the population improves each generation until an acceptably fit individual is produced.

GA experimental design. A series of five experiments (generations 1 to 5) were performed at 2-week intervals. The number of generations was limited due to the constraints of herbage production over the normal growth season. Within each generation, 50 treatments selected by the GA program were studied. Each treatment consisted of each of the eight different additives ("genes"; additives A to H) at a level between 0 and 5 (Table 1). Three replicates of control silage (the same herbage but without any additives) were also prepared.

The GA software used was written by R.J.G. For the GA we used a total population consisting of 100 individuals, although each new generation consisted of only 50 individuals (treatment combinations). The GA was initiated with a random population of 50 individuals. A second generation of 50 individuals was created, 20 by single-point mutation and 30 by single-point crossover. The 100 individuals from generations 1 and 2 were then pooled and sorted by fitness. The 50 fittest individuals were then used as parents for generation 3, and the 50 least fit individuals were discarded. This process was repeated for subsequent generations (Fig. 1). The 50 treatments in each generation were split into five subpopulations (demes), each of which contained 10 individuals. Reproduction occurred only between members of the same deme, but after three generations the best 10% of the population as a whole were copied into each deme to simulate migration. It has been shown that this migration mechanism significantly improves the efficiency of a GA search (60). The overall strategy used in the present study is shown in Fig. 1.

Preparation and analysis of full-term silages. Silage was prepared as described above by using the same herbage plots that were used for the GA experiments; the plots were cut after a 4-week period of regrowth. A total of five treatments were used. These treatments included the three additive combinations which produced the highest fitness values in the last (fifth) generation of the GA series. A treatment control containing a commercially available additive (Rapid Act; Biotal Ltd., Cardiff, United Kingdom) and an untreated silage control (water only) were also prepared. The herbage was separated into five 6-kg portions, and each portion was sprayed with 120 ml of a treatment preparation and mixed well. The treated herbage was then divided into 18 100-g portions and three 1-kg portions. The 100-g samples were packed into glass tubes as described above. The 1-kg samples were packed into glass preserving jars (Weck, Wehr Offingen, Germany) equipped with air locks.

All of the tubes and jars were incubated in a temperature-controlled room at 18°C for up to 100 days. Tubes were opened after six intervals (1, 2, 4, 14, 60, and 100 days). At each time point three replicate tubes for each of the five treatments were opened and analyzed as described above to determine the DM, content, the lactate content, the pH, and the free amino acid content. The microorganisms present (LAB, enterobacteria, and yeasts) were also counted by the methods described by Merry et al. (30). The 1-kg silage jars were opened after 100 days. The following analyses were performed with these full-term samples in addition the analyses described above: aerobic stability was measured by determining the increase in temperature after silage was aerated (11), and in vitro digestibility of the silage was assessed by measuring gas production with the automated pressure evaluation system (13, 50). The culture fluid pH, DM loss, and volatile fatty acid production were measured at the end of the fermentation period by methods described by Merry et al. (32).

Statistical analysis. Analysis of variance was performed by using the multivariate analysis function of Genstat 5 (51). Treatments were compared by calculating the least significant difference by using the standard error of the difference (supplied by Genstat) and the *t* value at the appropriate degrees of freedom.

RESULTS

Determination of optimal silage fermentation time. The aim of the initial experiment was to assess how soon after the start of fermentation valid measurements of silage fermentation characteristics and quality could be obtained. Three silages (one untreated control silage, one silage treated with *L. plan-*

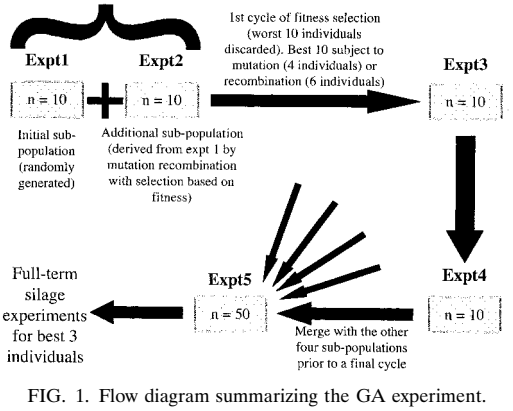

FIG. 1. Flow diagram summarizing the GA experiment.

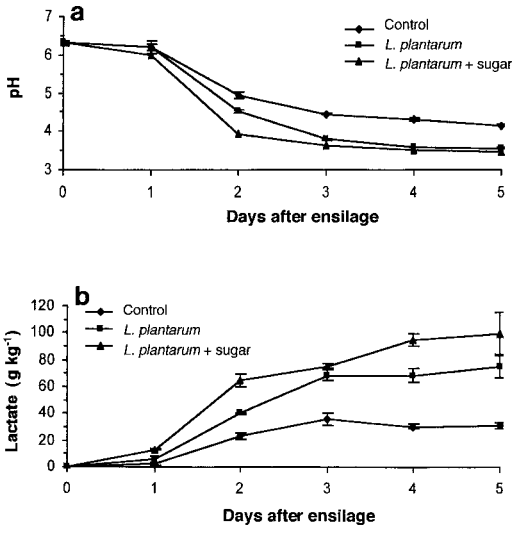

FIG. 2. Time course experiment performed to select a suitable period for harvesting the 100-g silos. (a) Change in pH over a 5-day period. (b) Change in lactate content over a 5-day period.

tarum inoculant, and one silage treated with *L. plantarum* plus sugars; additives at level 4 [see Table 1]) were prepared. The changes in pH values and lactate concentrations over the first 5 days of ensilage are shown in Fig. 2. The decrease in pH and the increase in lactate concentration were faster and greater in the inoculant-treated silages than in the control. After 48 h the changes in pH values and lactate levels were large enough so that accurate measurements could be obtained, and the differences correlated well with those observed over longer periods.

Using GAs for optimization is an iterative process, and successive generations of experiments result in progressively improved fitness values. Since the time scale of a full-term silage fermentation is typically 3 to 6 months and there is limited availability of fresh forage (which is available from May to October in the United Kingdom), assessment of fitness parameters in order to optimize additive combinations with mature (full-term) silages is effectively limited to an annual cycle. The occurrence of significant changes within 48 h of ensilage (which is thought to be the most critical period in terms of reducing adverse plant and microbial enzymatic activity and minimizing deterioration in herbage quality [32, 48]) results in a significant practical benefit since a larger number of GA generations can be examined each season.

Nevertheless, cutting, treating, and packing of the herbage at the start of the ensilage process and then unpacking and analysis of the minisilos are time-consuming activities. Therefore, based on 48-h silage fermentation data, we concluded that a turn-around period of 14 days would be feasible for each GA generation; this would allow up to six or seven generations per season.

Design of GA parameters. We used three freeze-dried LAB preparations, two fresh LAB cultures, two enzymes, and a sugar mixture to represent the diversity of additives currently available commercially in the United Kingdom. These preparations were added at six different levels (levels 0 to 5), as shown in Table 1. The number of possible combinations, 6^8 (1,679,616), was large enough to provide a complex fitness landscape within which the heuristical prowess of the GA could be tested. Level 3 represented a typical rate of application in standard agricultural practice for these types of additives; two higher levels and three lower levels (including zero) were also used. Each individual encoded the level of each of the eight additive components as an integer in the range from 0 to 5. The "chromosome," therefore, comprised an array of eight integers, each of which represented the amount of one component in the additive mix.

GAs function efficiently (in terms of optimizing the desired additive combination) only if suitable fitness parameters are chosen. In the absence of any detailed data concerning the likely shape of the response surface, some modelling was conducted in silico in order to determine the structure of a GA that was likely to improve the fitness in a very limited number of generations. The limiting factors were the number of logistically feasible fitness assessments per generation (approximately 50) and the number of generations that could be studied in a single growing season (approximately six). Within these constraints, the size and number of subpopulations, the size and frequency of migration between subpopulations, the mutation rate, and the crossover rate were examined in order to determine the most efficient strategy for the GA. The fitness function used in this parameterization stage was a mathematical expression of dimensionality 8, the same as the number of components in the silage additives. This expression was chosen so that there were independent variables and variables whose values were linear and nonlinear combinations in order to approximate the potential interaction complexity of the silage additive components, as follows: fitness = $1/\{1 + G1 + (5 - G2) + [1/(1 + G3/G4)] + (G5 - G6) + [1 + \sin(G7)]/2 + G8 \times G8\}$. The GA parameters selected for actual implementation were those that gave the best fitness value for an average of 10 replicate runs.

For the silage fermentation (logistically constrained as described above), we decided to determine fitness relative to control silage (herbage ensiled without any additives; i.e., the level of each additive was zero) in each experiment, and select for a large decrease in pH, a large increase in lactate content, and for low free amino acid levels; each of these factors was given a particular weighting. This strategy also took into account the likelihood that the quality of the herbage would change during the summer (Table 2). A fitness value was as-

TABLE 2. Composition of the herbage used in the ensilage experiments

Time course or generation	Cutting date (day-mo-yr)	DM concn (g kg of fresh matter^{-1})	pH	WSC concn (g kg of DM^{-1})	Total N concn (g kg of DM^{-1})	Buffering capacity (meq kg of DM^{-1})
5-Day time course	20-5-98	192.1 (0.14)[a]	6.31 (0.05)	165.7 (5.1)	25.8 (0.4)	218.03 (13.5)
Generation 1	16-6-98	162.6 (0.19)	6.23 (0.05)	171.3 (5.9)	29.1 (0.4)	191.98 (9.94)
Generation 2	30-6-98	142.2 (0.11)	6.05 (0.00)	173.3 (9.0)	25.1 (0.5)	201.36 (9.67)
Generation 3	28-7-98	144.1 (0.15)	6.00 (0.03)	154.0 (5.6)	27.3 (0.8)	170.14 (4.28)
Generation 4	11-8-98	144.5 (0.17)	6.19 (0.02)	114.7 (2.3)	32.7 (0.4)	188.81 (3.22)
Generation 5	25-8-98	150.5 (0.06)	6.05 (0.01)	97.0 (3.6)	39.1 (0.5)	184.39 (9.30)
100-Day time course	16-9-98	145.4 (1.94)	6.04 (0.04)	124.0 (5.0)	32.2 (0.3)	205.75 (14.27)

[a] The values are means (standard deviations) based on three replicates.

signed to each of the treatments based on the results of the pH, lactate concentration, and free amino acid content analyses and on the relative economic cost of the treatments, which was calculated by using the following equation:

$$\text{fitness} = \frac{1}{1 + [\text{pH wtg} \times (\text{pH/ctrl pH})] + [\text{lac wtg}/(\text{lac/ctrl lac})] + [\text{aa wtg} \times (\text{aa/ctrl aa})] + (\text{cost}/20)}$$

where wtg is weighting, ctrl is control silage, lac is lactate content, and aa is amino acid content. Changes in these three parameters were expressed as ratios relative to the control silage for each set of experiments (i.e., the pH ratio was calculated by dividing the pH for each treatment combination by the mean pH for the three control silages in each experiment). There are obvious interactions between these fitness parameters, since lactic acid level is closely related to the decrease in pH, which in turn influences the extent of protein breakdown. Lactate accumulation is the easiest parameter to measure accurately and the parameter in which the greatest change was anticipated. Furthermore, the decrease in pH is also related to the buffering capacity of the crop (which is known to vary depending on the composition of the herbage). Therefore, the weighting given to the lactate level was increased to 4 (the lactate level contributed 40% to the fitness value instead of 33%, the level that it would have contributed if the three factors had been given equal weighting), the weighting given to the decrease in pH was reduced to 2.5, and the weighting given to the free amino acid level was 3.5. (aa wtg). Thus, the fitness value for the control silages (no additives) was 0.091 (1/[1 + 10]), and improved silages had higher fitness values.

For any optimization method to provide useful results, not only must realistic parameter ranges be chosen, but some economic cost element must also be incorporated. In the absence of any cost penalties, it is likely that the GA will select higher levels of some or all additives than are actually required (assuming that no treatments have a negative effect on fitness), which can result in high-quality but uneconomical or impractical additive combinations. The importance of realistic cost function criteria has long been appreciated by users of GA methods (6, 18, 33), and a poor choice can hamper the heuristical efficiency of the GA approach. For this experiment, determining the cost function associated with each additive was simple because the retail costs of all of the additives were very similar (ca. $1.5 per ton at the standard inoculation rates used in the United Kingdom, although this value does not reflect additive production costs). The cost of each treatment combination was calculated by dividing the sum of the values for the treatment levels in the silage by 20, so that the maximal cost factor (i.e., when all additives were added at level 5) was 2 ([5 × 8]/20). In practice, the cost function accounted for 10 to 20% of the total fitness value.

GA experiment. The DM content, pH, WSC content, and total nitrogen (N) content values for the herbage used in each GA experiment are shown in Table 2. The DM content of the herbage used was low throughout the study (14 to 16% of the fresh matter content), and the pH was between 6.00 and 6.23. The WSC levels decreased during the summer (particularly in generations 4 and 5), while the total N levels increased.

Logistical constraints limited the population size to 50 treatments per generation. The additive formulations used for the first generation were generated randomly. After pH, lactate, and free amino acid values had been determined for each minisilo and the fitness of each treatment had been calculated, the values were entered into the GA program in order to select the parents for the next generation of treatments on the basis of the fitness values. All of the treatment combinations improved the fermentation rate and quality of the silage after 2 days of ensilage compared to the untreated controls.

Between the first experiment and the fifth experiment there were increases in the mean, maximal, and minimal fitness values (Table 3; Fig. 3a), despite the fact that the quality of the herbage decreased in the fourth and fifth GA experiments (as shown by the lower WSC levels in the herbage [Table 2] and the reduced lactate levels in the uninoculated control silages [Table 3]). Between generation 1 and generation 5 the mean fitness value increased from 0.119 to 0.122. The improvement in silage quality was most graphically shown by the change in lactate levels compared to the control silage (Fig. 3b); the mean concentration in generation 5 was 3.21 times greater than the mean concentration in the control (which represented an almost twofold increase compared with the mean concentration in generation 1). The best treatment combination in generation 5 in terms of lactate levels (index no. 2540234; fitness value, 0.119) resulted in a lactate ratio that was 4.56 times greater than the control.

The patterns for pH and free amino acid content (in terms of both absolute values and ratios compared to the control) were less clear. In particular, the concentration of free amino acids and the ratio compared to the control increased (results which were opposite of those desired) in successive experiments. However, this may have been a result of the steady increase in total N levels in the initial herbage (which were 35% higher in experiment 5 than in experiment 1 [Table 2]). The inconsistent pattern of pH decreases in the uninoculated control silages may have been influenced by the buffering capacity of the herbage (Table 2), which varied during the season. However, in all of the treated silages, the pH values were less than 4; thus the environment was sufficiently acidic to inhibit potential spoilage organisms, such as enterobacteria and clostridia. The total amount of additive used for each treatment (i.e., total cost) varied little through the five generations, ranging from 21.5 to 22.3 (Table 3). Thus, the cost element of fitness did appear to prevent progressively higher levels of additive from being selected. A product moment correlation analysis of the levels of each additive and the fitness levels did not yield statistically significant coefficients of correlation.

Full-term silage trials. For the fitness components in the GA experiment we relied on analyses of silages performed after 48 h. However, related parameters (which for logistical reasons could not be assessed during the GA experiment) are important for validating the GA choice of additive combinations. Therefore, the three additive combinations that gave the highest fitness values in the fifth (last) generation of the GA experiment (index no. 02235404, 32055034, and 43242002) were used to conduct a full-term (100-day) silage fermentation experiment, and a range of parameters were measured at intervals. These parameters included those measured during the GA experiments, as well as other indicators of silage fermentation quality, such as volatile fatty acid and ammonia levels, resistance to aerobic spoilage, and in vitro digestibility, as well as numbers of LAB and spoilage microbes. Larger-scale (1-kg) jar silos were also set up and analyzed in the same way after 100 days.

The three test silages generated in the GA experiment were compared to a control (uninoculated) silage and to a silage made with a commercial additive (RapidAct; Biotal Ltd.). In both the 100-g tube silos and the 1-kg jar silos, two of the three GA-selected silages (5;1.07 and 5;3.10) were superior to the control and the RapidAct-inoculated silages in terms of de-

TABLE 3. Results of the GA grass silage experiments[a]

Generation	Value	pH	Lactate concn (g kg of DM^{-1})	Amino acid concn (mol kg of DM^{-1})	pH ratio	Lactate ratio	Amino acid ratio	Fitness	Cost
1	Mean	3.83 a	70.34 a	0.242 a	0.769 a	1.754 a	0.608 a	0.119 a	21.5
	Minimum	3.69	47.07	0.130	0.741	1.174	0.327	0.097	13
	Maximum	3.98	87.74	0.373	0.799	2.187	0.937	0.137	32
	Control	4.98	40.11	0.398					
2	Mean	3.66 b	77.14 b	0.260 ab	0.895 b	1.670 b	0.720 b	0.108 b	21.5
	Minimum	3.53	52.36	0.123	0.861	1.134	0.341	0.092	13
	Maximum	3.92	92.72	0.433	0.956	2.008	1.199	0.129	30
	Control	4.10	46.18	0.361					
3	Mean	3.56 c	72.21 a	0.270 b	0.841 c	1.501 c	0.743 bc	0.106 b	22.3
	Minimum	3.44	51.65	0.157	0.812	1.073	0.432	0.089	10
	Maximum	3.94	98.35	0.392	0.930	2.044	1.078	0.118	31
	Control	4.24	48.12	0.364					
4	Mean	3.73 d	78.48 b	0.316 c	0.828 d	2.178 d	0.820 d	0.113 c	22.1
	Minimum	3.61	53.76	0.141	0.800	1.492	0.367	0.098	13
	Maximum	3.94	102.87	0.426	0.874	2.854	1.106	0.136	31
	Control	4.51	36.04	0.385					
5	Mean	3.73 d	68.44 a	0.409 d	0.850 e	3.210 e	0.780 cd	0.122 d	21.6
	Minimum	3.61	32.26	0.236	0.822	1.512	0.451	0.103	12
	Maximum	3.94	97.37	0.502	0.897	4.565	0.961	0.140	30
	Control	4.39	21.33	0.523					

[a] Different letters after values in a column indicate that the values were significantly different ($P > 0.05$). Maximum and minimum values for individual treatments in each generation are also shown. The pH, lactate, and amino acid ratios were calculated by comparing values with the control silage values in each experiment. Fitness values were calculated as described in the text. Cost was calculated by adding the eight inoculant values. The GA was set to maximize lactate levels while the pH and amino acid concentration were minimized.

crease in pH, lactate level, and free amino acid level (Table 4). The differences in the pH and lactate values were greater during the first 2 weeks of ensilage than after 60 and 100 days, while the free amino acid levels in the two GA-selected silages remained significantly lower than the control and RapidAct silage levels throughout. This is consistent with the known effect of rapid acidification on protein preservation (12, 20).

An analysis of the volatile fatty acids revealed that neither butyrate (an indicator of spoilage by *Clostridium* spp.) nor propionate (often considered desirable due to its antimycotic activity which reduces aerobic spoilage) accumulated at a significant level (data not shown). Two of the three GA-selected silages (5;1.07 and 5;3.10) had lower levels of acetate (<12.5 g kg^{-1}) and free ammonia than the control and RapidAct silages (Table 4). The microbiological analysis showed that the GA-selected silages contained higher levels of LAB after 1 and 2 days, but thereafter LAB levels were similar in all silages (data not shown). Members of the family *Enterobacteriaceae* were detected after 1 or 2 days of ensilage in all silages but not thereafter (Table 4). Aerobic stability and in vitro digestibility tests revealed no significant differences, and all of the silages performed well (data not shown). The third GA-selected silage (5;4.07) did not perform well, as judged by the parameters used to determine silage quality (Table 4). However, this silage did have a higher level of lactate after 2 days than the other silages (which was probably the reason why this additive combination was selected by the GA), but it appeared to deteriorate during later stages of fermentation. The levels of acetate and ammonia were higher in this silage during later stages of fermentation.

DISCUSSION

The use of a GA to select ingredients for silage additives is a novel approach which permits a wide range of additive permutations to be screened in a rational manner, a feat which is impractical when more established optimization methods are used. Despite the long history of GAs and their current widespread use, there have been only a few examples of using GAs in fermentation technology. For instance, recently, Weuster-Botz et al. (56–59) and Zuzek et al. (67) used GAs to study medium optimization (for instance, to maximize hydrocortisone Δ^1-dehydrogenase activity in *Arthrobacter simplex* cultures in a synthetic medium [58]). In our proof of principle experiments we examined whether it is feasible to use GAs to study an experimental system that is significantly more complex. Not only were most of the additives viable bacteria (in contrast to the synthetic medium components used in the studies men-

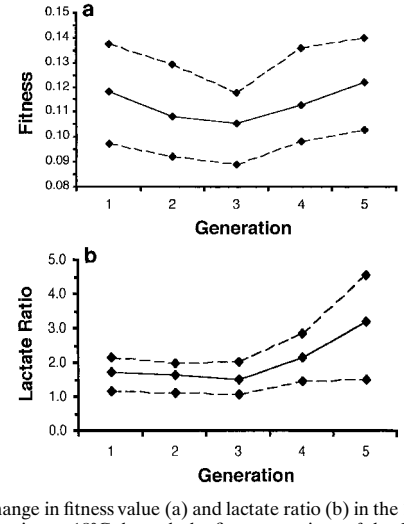

FIG. 3. Change in fitness value (a) and lactate ratio (b) in the 100-g silos after 2 days of incubation at 18°C through the five generations of the GA experiment. The dotted lines indicate the maximal and minimal values.

tioned above), but the quality of the substrate itself (i.e., the herbage being ensiled) was variable (Table 2). Furthermore, the nature of the fitness function was significantly more complex and involved three experimental measurements, as well as a cost function, whereas in previous experimental GA approaches the researchers did not use a cost function and used simpler methods (e.g., biomass or product yield) to determine fitness. Despite the fact that the quality of the herbage decreased as the experiments progressed (in particular, there were progressive decreases in WSC levels), there were nevertheless increases in the mean and maximum fitness values between generation 1 and generation 5.

Optimization of silage additives in a rational manner is a task fraught with potential problems. First, the objective definition of good silage is not always clear. It is well known that rapid growth and acidification by homofermentative LAB is the basic aim of the silage fermentation so that sugars are efficiently converted to lactic acid, leading to suppression of nonbeneficial microbes (by both direct competition and lowering of the pH). However, other parameters that are more difficult to measure directly must also be considered; these include preservation of plant proteins in a digestible form, aerobic stability of the silage during the feed-out phase, and nutritional quality in relation to intake levels and livestock productivity (27).

During ensilage a number of criteria may be used to assess the progress of the fermentation. The rate and extent of the decrease in pH reflect the homolactic efficiency of the LAB in the silage, while the lactate level provides a direct measure of this efficiency. A rapid increase in the lactate level results in a decrease in the pH to a value less than 4.0 which inhibits the activity of potential spoilage microbes, such as *Clostridium* spp., members of the *Enterobacteriaceae*, and pathogenic *Listeria* spp. (15, 40, 49). Lactate level was the experimental parameter which improved most significantly during the GA experiments. It was also the parameter which could be most accurately measured (Table 3), partly because lactate is not present in fresh herbage and also because the lactate level is a direct measure of the activity of homofermentative LAB. If this parameter had been the only fitness parameter used, it is very likely that the overall improvement in fitness through the five generations would have been greater. Including the decrease in pH was to some extent redundant, since only low levels of other organic acids or ammonia (<9 g of acetic acid kg^{-1} and <1.5 g of NH_3 kg^{-1} after 2 days [Table 4]) were present during the early stages of ensilage. We also found that all of the inoculated silages had pH values less than 4.0 by 2 days; these values were well below the critical level necessary to inhibit proliferation of spoilage microbes (44, 54).

Rapid acidification has also been found to be important in minimizing proteolysis during ensilage (27). The main products of protein breakdown are amino acids and ammonia, and the proportion of each depends on the extent of amino acid metabolism by silage microbes (12, 27). Using an additive which decreases the pH more rapidly should therefore reduce proteolysis and the level of free amino acids (which was used in this study as an indirect but convenient and rapid way to measure proteolysis). However, neither the metabolism of proteins in silage by microbes and plant enzymes nor the direct effects of acidification on protein breakdown have been studied in detail, so it is not clear how closely the free amino acid pool in silage reflects the level of protein preservation (1, 12, 22, 35).

There was a steady increase in the total N content of the herbage through the five generations. This may explain why the absolute mean level of free amino acids (and to a lesser extent the ratio of free amino acids compared to the control silage)

TABLE 4. Time course analysis of full-term silages

Ensilage period (days)	pH				Total lactate concn (g kg of DM^{-1})				Free amino acid concn (mol kg of DM^{-1})				Concn of members of the *Enterobacteriaceae* (log_{10} CFU g^{-1})				Acetate concn (g kg of DM^{-1})				Ammonia concn (g kg of DM^{-1})			
	5;1.07	5;3.10	5;4.07	Rapid Act	5;1.07	5;3.10	5;4.07	Rapid Act	5;1.07	5;3.10	5;4.07	Rapid Act	5;1.07	5;3.10	5;4.07	Rapid Act	5;1.07	5;3.10	5;4.07	Rapid Act	5;1.07	5;3.10	5;4.07	Rapid Act
1	4.26	3.92	4.83	5.40	45.87	57.01	29.22	13.62	0.159	0.087	0.206	0.191	5.66	4.63	3.95	6.39	2.94	4.34	1.98	4.00	0.65	0.53	0.64	0.92
	(0.01)	(0.02)	(0.11)	(0.03)	(4.32)	(3.52)	(0.43)	(0.88)	(0.008)	(0.023)	(0.032)	(0.017)	(4.48)	(4.24)	(3.56)	(5.98)	(1.38)	(0.41)	(0.27)	(1.85)	(0.07)	(0.04)	(0.13)	(0.04)
2	3.59	3.58	3.83	3.86	96.2	82.17	98.62	46.09	0.170	0.170	0.220	0.194	0	2.82	0	3.48	6.86	5.92	8.53	5.71	0.57	0.44	0.83	0.81
	(0.02)	(0.02)	(0.03)	(0.02)	(7.88)	(37.58)	(12.45)	(24.53)	(0.039)	(0.001)	(0.023)	(0.004)		(3.06)		(3.00)	(2.29)	(1.37)	(0.50)	(2.14)	(0.03)	(0.12)	(0.02)	(0.25)
4	3.53	3.54	3.73	3.71	3.91	116.43	118.36	97.67	100.47	62.46	0.233	0.260	0.241	0.286	0.357	0	6.43	4.90	9.51	7.98	8.39	0.63	0.62	0.92
	(0.02)	(0.02)	(0.04)	(0.02)	(0.02)	(18.56)	(11.93)	(9.17)	(16.34)	(13.01)	(0.044)	(0.047)	(0.002)	(0.036)	(0.015)		(1.02)	(1.02)	(2.53)	(0.37)	(1.28)	(0.02)	(0.06)	(0.04)
14	3.43	3.45	3.58	3.62	3.62	91.06	114.58	95.39	103.14	90.25	0.324	0.308	0.329	0.352	0.387	0	7.33	6.10	13.27	9.91	10.61	0.85	0.85	1.70
	(0.01)	(0.01)	(0.03)	(0.02)	(0.02)	(22.78)	(41.46)	(15.04)	(13.86)	(4.97)	(0.012)	(0.015)	(0.008)	(0.047)	(0.021)		(0.23)	(0.30)	(3.94)	(0.35)	(1.36)	(0.03)	(0.01)	(0.22)
60	3.40	3.37	3.65	3.74	3.51	165.78	164.22	157.20	138.28	104.35	0.708	0.365	0.502	0.633	0.726	0	0	0	0	0	8.57	5.74	17.45	17.17
	(0.01)	(0.02)	(0.04)	(0.27)	(0.02)	(8.30)	(7.32)	(15.19)	(29.73)	(43.47)	(0.040)	(0.047)	(0.023)	(0.064)	(0.133)						(0.31)	(0.84)	(2.47)	(8.81)
100	3.52	3.52	3.73	3.64	3.59	159.74	152.33	139.65	150.38	150.77	0.698	0.349	0.498	0.626	0.625	0	0	0	0	0	11.03	10.51	21.06	16.45
	(0.01)	(0.05)	(0.04)	(0.03)	(0.01)	(1.01)	(15.17)	(2.70)	(3.26)	(4.77)	(0.035)	(0.037)	(0.054)	(0.140)	(0.106)						(0.26)	(0.98)	(3.09)	(0.86)
100 (jar)	3.54	3.57	3.98	3.78	3.75	152.91	155.64	80.55	137.70	144.91	0.321	0.316	0.318	0.663	0.715	ND[b]	ND	ND	ND	ND	12.47	9.78	59.27	18.77
	(0.01)	(0.01)	(0.12)	(0.01)	(0.03)	(2.64)	(0.59)	(32.22)	(2.64)	(21.12)	(0.050)	(1.07)	(0.051)	(0.106)	(0.047)						(2.26)	(0.59)	(35.97)	(2.70)

Ensilage period (days)	Ammonia concn (g kg of DM^{-1})											
	5;1.07	5;3.10	5;4.07	Rapid Act	Control	5;1.07	5;3.10	5;4.07	Rapid Act	Control	5;1.07	5;3.10	5;4.07	Rapid Act	Control
1	0.65	0.53	0.64	0.92	1.08	0.65	0.53	0.64	0.92	1.08	0.65	0.53	0.64	0.92	1.08
	(1.09)	(0.17)	(0.01)	(0.45)	(0.06)	(1.09)	(0.17)	(0.01)	(0.45)	(0.06)	(1.09)	(0.17)	(0.01)	(0.45)	(0.06)
2	0.57	0.44	0.83	0.81	1.42	0.57	0.44	0.83	0.81	1.42	0.57	0.44	0.83	0.81	1.42
	(0.03)	(0.01)	(0.22)	(0.25)	(0.13)	(0.03)	(0.01)	(0.22)	(0.25)	(0.13)	(0.03)	(0.01)	(0.22)	(0.25)	(0.13)
4	1.17	1.08	3.16	2.97	2.18	1.17	1.08	3.16	2.97	2.18	1.17	1.08	3.16	2.97	2.18
	(0.06)	(0.01)	(0.36)	(0.75)	(0.06)	(0.06)	(0.01)	(0.36)	(0.75)	(0.06)	(0.06)	(0.01)	(0.36)	(0.75)	(0.06)
14	1.43	1.49	3.29	2.85	2.41	1.43	1.49	3.29	2.85	2.41	1.43	1.49	3.29	2.85	2.41
	(0.07)	(0.05)	(0.17)	(0.14)	(0.44)	(0.07)	(0.05)	(0.17)	(0.14)	(0.44)	(0.07)	(0.05)	(0.17)	(0.14)	(0.44)
60	1.22	1.21	2.79	2.34	2.13	1.22	1.21	2.79	2.34	2.13	1.22	1.21	2.79	2.34	2.13
	(1.09)	(0.01)	(0.45)	(0.06)	(0.30)	(1.09)	(0.01)	(0.45)	(0.06)	(0.30)	(1.09)	(0.01)	(0.45)	(0.06)	(0.30)
100	0.85	0.85	1.70	1.82	1.98	0.85	0.85	1.70	1.82	1.98	0.85	0.85	1.70	1.82	1.98
	(0.03)	(0.01)	(0.22)	(0.25)	(0.13)	(0.03)	(0.01)	(0.22)	(0.25)	(0.13)	(0.03)	(0.01)	(0.22)	(0.25)	(0.13)
100 (jar)	1.17	1.08	3.16	2.97	2.18	1.17	1.08	3.16	2.97	2.18	1.17	1.08	3.16	2.97	2.18
	(0.06)	(0.01)	(0.36)	(0.75)	(0.06)	(0.06)	(0.01)	(0.36)	(0.75)	(0.06)	(0.06)	(0.01)	(0.36)	(0.75)	(0.06)

[a] The values are means (standard deviations). The following three silages were selected from the final generation of the GA experiments: 5;1.07 (index no. 02235404; fitness value, 0.133); 5;3.10 (index no. 32055034; fitness value, 0.133); and 5;4.07 (index no. 43242002; fitness value, 0.140). The RapidAct inoculant contained 3.73×10^5 CFU of freeze-dried *L. plantarum* per g of treated herbage, 3.88×10^5 CFU freeze-dried *P. pentosaceus* per g of treated herbage, 4,918 IU of amylase per g of product, 11,291 IU of β-glucanase per g of product, 2,567 IU of galactomannanase per g of product, and 19,088 IU of xylanase per g of product (information supplied by Biotal Ltd.). The control silage was treated with an equivalent volume of distilled water.

[b] ND, not determined.

increased through the five generations. A more direct measure of the percentage of the original herbage N preserved in a form which can be efficiently assimilated by livestock (i.e., "rumen-protected" protein [7, 41]) would provide a better fitness parameter.

The cost function used in our experiments accounted for between 10 and 20% of the total fitness value. A cost function was included in order to direct the GA towards efficient solutions (i.e., solutions resulting in high levels of acidification and reduced proteolysis without unnecessarily high additive levels). The presence of an effective cost function is known to be an important component of efficient GA design (6, 18, 33); otherwise, good but uneconomic additive combinations might be selected. Although the aim of the present series of experiments was to test the principle that GAs are a useful tool for selecting additive combinations (rather than for designing new additive formulations per se), a simple cost function was included in order to avoid excessive convergence on additive combinations that were not economically feasible. The fact that two of the three fittest silages in the last experiment contained at least one additive at the highest level (which would be difficult to achieve on a farm scale) suggests that a more drastic cost function (e.g., $cost^2$) might be appropriate. However, it must be borne in mind that our GA experiment was terminated after only five generations and that additional generations could have resulted in further increases in fitness and quite possibly convergence on additive combinations at lower levels.

Although the control (no additive) silages in the present study were inferior to additive-treated silages, in all of the silages some lactate accumulated and the pH decreased to less than 5.0 within 2 days. In the full-term control silages, the pH continued to decrease to values less than 4.0 and the lactate concentration increased to more than 100 g kg of DM^{-1} by 100 days (Table 4). It is perhaps not surprising that substantial differences in in vitro digestibility or aerobic stability between these silages and the additive-treated silages were not observed. However, under farm conditions, where there is often a significant delay between harvesting and the filling of the silo (i.e., the onset of anaerobic conditions), particularly if the herbage has a low DM content (i.e., $<20\%$) due to wet weather, the role of additives in initiating homolactic fermentation is more critical. Another incentive for the development of more advanced silage additives (particularly in the United Kingdom following the recent bovine spongiform encephalitis crisis) is the trend towards ensilage of legume crops with increased protein content in order to provide alternative sources of nitrogen (17, 23). The nature of these forage crops (which often have low WSC levels [typically <80 g kg^{-1}] and a high buffering capacity) is less conducive to efficient ensilage in the absence of additives (10, 45). The GA approach provides an efficient way to develop crop-specific additives by possibly incorporating parameters related to microbiological feed safety (e.g., reduction in the proliferation of coliform bacteria or pathogenic *Listeria* spp.) and nutritional quality when the fitness is calculated. Many LAB are known to produce bacteriocins (3, 8, 34) (e.g., pediocins are produced by *P. pentosaceus* [14]), which may inhibit pathogenic bacteria by more specific mechanisms in addition to contributing to silage acidity.

In the present study we found that suitable additive combinations for improving silage quality can be selected by using a GA to guide the experimental process, even in the face of problems due to the variability of the herbage over the growing season. Longer-term work may now be done to select optimal treatments by using herbage obtained over several seasons.

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