

Chapter Three

3. Methods

3.1 Microbial techniques

3.1.1 Microbial analysis of silage

The protocol for the microbial analysis of silage is a standard operating procedure provided by Ecosyl Products Ltd. The silage sample (30 g) was mixed with ¼-strength Ringers solution (270 ml) in a stomacher bag and stomached (3 minutes). A 1 ml volume of the resultant slurry was removed and diluted with ¼-strength Ringers solution in 10-fold serial dilutions in sterile plastic universal bottles. The CFU count of yeasts, moulds, lactic acid bacteria, acetic acid bacteria and spores were made using selective media in Petri dishes, as detailed below. Dishes were prepared from at least three different members of the dilution series, and three replicates were made at each dilution. The dilution producing 30–300 CFU per plate (15–150 in the case of moulds) was analysed with a colony counter, and the result expressed as an average of the three replicates.

For the estimation of mould and yeast CFUs, molten ME agar was prepared and cooled to 50°C. A solution of streptomycin sulphate (6 mg/ml) and penicillin G (6 mg/ml) was prepared and sterilised by filtration (0.2 µm). This was added to the ME agar (5 ml/l, to give a final concentration for each of 30 mg/l), and mixed thoroughly. For yeast CFU count, an aliquot (100 µl) of the appropriate dilution was pipetted into a Petri dish. ME agar + antimicrobials (approximately 25 ml) was poured over and swirled to mix. Dishes were left to harden, then incubated (30°C) for two days. For mould CFU count, Petri dishes were prepared containing solid MEA + antimicrobials (25 ml). An aliquot (100 µl) of the appropriate dilution was pipetted onto this and spread evenly over the surface using a sterile plastic spreader. The dishes were incubated (25°C) for four days,

and mycelial colonies with an obvious mould-like morphology (*i.e.* filamentous) were counted.

For the estimation of lactic acid bacteria CFUs, molten MRS agar was prepared and cooled to 50°C. Cycloheximide solution (20 mg/ml) was filter sterilised, and 5 ml added to the MRS (final concentration 100 mg/l), which was then mixed thoroughly. An aliquot (100 µl) of the appropriate dilution was pipetted into a Petri dish. MRS + cycloheximide (approximately 25 ml) was poured over and swirled to mix. Dishes were left to harden, then incubated (30°C) for two days.

For the estimation of acetic acid bacteria CFUs, molten YE agar was prepared and cooled to 50°C. A solution of penicillin-G (0.31 mg/ml) and cycloheximide (20 mg/ml) was prepared and sterilised by filtration (0.2 µm). This was added to the YE agar (5 ml/l), to give a final concentration of 1.54 mg/l penicillin-G and 30 mg/l cycloheximide), and mixed thoroughly. Petri dishes were prepared containing YE agar + antimicrobials (25 ml), onto which was pipetted an aliquot (100 µl) of a member of the dilution series. This was spread evenly over the surface using a sterile plastic spreader, and the dishes incubated (30°C) for three days.

For the estimation of spore CFUs, the bottle containing the desired member of the dilution series was placed in a water bath (70°C). A dummy bottle containing a thermometer and an equivalent volume of water was also placed in the bath, and the temperature monitored. The bottles were allowed to reach 70°C, and were left at that temperature for 10 minutes. They were then removed, an aliquot (100 µl) was pipetted onto the surface of a Petri dish containing nutrient agar, and this was spread evenly over the surface using a sterile plastic spreader. Dishes were incubated (30°C) and the colonies formed were counted after three days.

3.1.2 Estimation of microbial colony forming units in liquid culture

A 1 ml volume of the culture was removed and diluted with ¼-strength Ringers solution in 10-fold serial dilutions in sterile plastic universal bottles. 100 µl of selected members

of the dilution series (those that were expected to contain 300–3,000 CFU/ml) were pipetted onto the appropriate agar medium (MRS for LAB, ME for yeast). Where a co-culture of LAB and yeast was to be analysed, cycloheximide was added to the MRS (final concentration, 30 mg/l). Dishes were prepared from at least three different members of the dilution series, and three replicates were made at each dilution. The dilution producing 30–300 CFU per plate was analysed with a colony counter, and the result expressed as an average of the three replicates.

3.1.3 Isolation of lactic acid bacteria from silage

A silage sample (10 g) was added to ¼-strength Ringers solution (50 ml) and shaken for 30 minutes at 30°C. 10-fold and 100-fold serial dilutions were prepared from this and 20 µl plated onto MRS agar or, in later work, on Rogosa agar (Rogosa agar was used in later work because it suppressed the growth of non-acid tolerant bacteria, such as *Bacillus* spp). Whichever medium was used, cycloheximide (100 mg/ml) was added to inhibit fungal growth. Plates were incubated (30°C, 48 hours), and colonies with a *Lactobacillus*-like morphology (white, smooth, convex) were streaked onto individual Petri dishes containing MRS agar from which, after incubation (30°C, 48 hours), 1 colony was subcultured into MRS broth. Isolates were examined microscopically, and Gram-positive non-spore-forming rods were frozen in Hogness buffer for future use.

3.1.4 Storage of strains in frozen culture

A colony of the desired strain was picked from an agar plate and grown to late log phase in an appropriate medium (MRS for LAB strains, ME for fungal strains). LAB strains were mixed with Hogness buffer (1 part culture : 9 parts buffer). Fungal strains were mixed with yeast preservation mix (1 part culture : 1 part preservation mix). The mixture was dispensed into replicate vials (1.5 ml plastic screw-topped tubes) and frozen at -70°C. Vials destined for long term storage were retained at -70°C, whilst vials for regular use as starter culture inocula were removed to storage at -20°C.

3.1.5 Preparation of starter cultures

Frozen stock cultures were defrosted in a 37°C water bath. 100 µl was dispensed into an appropriate medium (MRS for LAB, ME for fungi, YE for AAB, 10 ml in a 25 ml glass screw top universal bottle). Bottles were incubated overnight at 30°C.

3.2 Determination of buffering capacity

Aliquots (250 ml) were titrated with 10 M HCl, and the effect on the pH of each aliquot added was recorded. The van Slyke buffering capacity was calculated using the method of Dawson *et al.*, 1986:

$$\beta = -\Delta A \div \Delta \text{pH}$$

Where $\Delta A = \text{H}^+$ added (moles/litre)

3.3 Determination of microbial growth capacity in novel media.

Starter cultures (Section 3.1.5) of the desired organisms were prepared. Aliquots (20 µl) were inoculated into the test medium (20 ml) in a screw-topped glass bottle. Bottles were placed at 30°C for 48 hours, and analysed for visible signs of growth.

3.4 Determination of acidifying capacity in SGM

Starter cultures (Section 3.1.5) in MRS were prepared of the test organisms. Aliquots (20 µl) were inoculated into SGM (20 ml) and incubated for 24 hours (30°C), after which time the pH was measured. There were six replicates, and overall differences were analysed by one-way analysis of variance. If a significant effect was seen, pairwise differences were analysed by Students t-test.

3.5 Assessment of antifungal activity

3.5.1 2 stage agar overlay assay for antifungal activity

When an aerobic screen was to be conducted: a starter culture (Section 3.1.5) of the test LAB was prepared, and an aliquot (20 μ l) was pipetted onto a Petri dish containing SSM (15 ml). The LAB culture was spread over an area of 25 cm². Three replicate dishes were prepared from each culture, and three sterile dishes were incorporated into each experiment to act as negative controls. The dishes were incubated (24 hours, 30°C).

The Petri dishes were then overlaid with a sterile 7 cm disc of filter paper (Whatman No. 2), followed by a layer of SSM (15 ml, at 45°C) containing a suspension of the indicator yeast (1.3 ml/l). Dishes were incubated (30°C) until yeast on the negative control had fully grown (about 48 hours). Growth of yeast over the test LAB was scored on an abstract scale:

- X No effect on yeast growth
- + Some observable inhibition
- ++ Some areas devoid of yeast growth
- +++ Complete inhibition of yeast growth on areas directly over the LAB

When an anaerobic screen was to be conducted: the protocol used was essentially the same as an aerobic screen, except that the agar used was SGM, and dishes were kept in anaerobic conditions during the incubation period.

3.5.2 1 stage agar overlay assay for antifungal activity

This was a development of the 1 stage assay (Section 3.5.1). A starter culture (Section 3.1.5) of the test LAB was prepared, and an aliquot (20 μ l) was pipetted onto a Petri dish containing SGM (15 ml). The LAB culture was spread over an area of 25 cm² in

the centre of the plate. Two replicate dishes were prepared from each culture, and 11 strains were tested in each experimental run. Each run of the experiment included a negative control, which consisted of a pair of dishes prepared with *Lactobacillus plantarum* MTD/1. The inoculated dishes were incubated anaerobically (30°C). When the assay was to be used as part of the screening protocol (Section 5.2), a 24 hour incubation was used (30°C). In later work, a 48 hour incubation was used, as it was found that this allowed a greater expression of the antifungal activity.

The Petri dishes were then overlaid with a sterile 7 cm disc of filter paper (Whatman No. 2), followed by a layer of SSM (15 ml, at 45°C) containing a suspension of the indicator yeast (1.3 µl/ml). Dishes were incubated (30°C) until yeast on the negative control had fully grown (about 72 hours). Growth of yeast over the test LAB was scored on an abstract scale:

- X No effect on yeast growth relative to MTD/1 (negative control)
- + Some observable inhibition relative to control
- ++ At least 2 cm² devoid of yeast growth
- +++ Complete or nearly complete inhibition of yeast growth on areas directly over the LAB

3.5.3 Well diffusion assay for antifungal activity

The appropriate agar was prepared and cooled to 50°C in a water bath. A starter culture (Section 3.1.5) of the test yeast was added (1 µl/ml). The agar was swirled to mix, aliquots (25 ml) were dispensed into sterile plastic Petri dishes, and allowed to harden. Wells (6 mm, up to 4 per dish) were cut into the agar with a cork borer and filled with the test liquid. Dishes were incubated (30°C, 72 hours), and assessed at least daily for yeast growth.

3.5.4 Agar plug method for assessing antifungal activity

A starter culture (Section 3.1.5) of the test LAB was prepared, and an aliquot (20 µl) spread over the surface of SGM agar (15 ml) in a Petri dish. Dishes were incubated anaerobically for 24 hours, 30°C. An agar plug (15 mm diameter) was removed from the LAB culture with a cork borer and placed onto Petri dishes containing SSM agar inoculated with the indicator yeast (15 ml agar, 1 µl/ml yeast). Dishes were incubated (30°C, 72 hours), and assessed at least daily for indications of yeast inhibition.

3.5.5 Sealed plate method for detecting volatile antifungal activity

This is based on the method used by Fiddaman & Rossall (1993) for detecting volatile antifungals produced by *Bacillus subtilis*.

A starter culture (Section 3.1.5) of the test bacteria was prepared, and an aliquot (20 µl) was spread over a Petri dish containing SGM agar (15 ml). The plates were incubated anaerobically (30°C, 48 hours).

A starter culture (Section 3.1.5) of the indicator yeast, *S. exiguus*, was prepared, and an aliquot (20 µl) was spread over a Petri dish containing SSM agar (15 ml). The lids were removed from both plates. The dish containing the test bacteria was inverted and placed over the dish containing the indicator yeast. The two dishes were sealed with parafilm and incubated (30°C) until yeast growth was observed on the surface of the SSM plate.

The time taken for the yeast to grow was compared with control dishes containing sterile SGM, and with dishes containing SGM seeded with *L. plantarum* MTD/1. Any differences were noted.

3.5.6 Liquid co-culture of test LAB and indicator organisms

Starter cultures (Section 3.1.5) of the test LAB were prepared in MRS. Prewarmed SGM (30°C, 200 ml in a 300 ml screw-topped bottle) was inoculated with the test starter cultures (0.2 ml), and incubated with shaking (120 rpm, 24 h, 30°C).

Half of the resultant culture (100 ml) was added to prewarmed SSM (100 ml, 30°C) in a baffled conical flask (500 ml capacity), loosely plugged with cotton wool. To this was added an inoculum (0.1 ml) of an indicator organism starter culture (Section 3.1.5). The mixed culture was incubated with shaking (120 rpm, 30°C). Samples (5 ml) were taken for the measurement of pH, optical density, or CFU count, as required.

3.5.7 Assay for catalase activity

This method is that described by Sigma for the assessment of catalase activity (Anon, 1996). The enzyme solution (Solution A) consists of the enzyme in phosphate buffer (2 mg/ml enzyme, 0.01 M PO₄, pH 5). The substrate (Solution B) consists of 20% H₂O₂ in phosphate buffer (approximately 0.1 ml in 50 ml buffer, 0.01 M PO₄, pH 5). The exact amount of H₂O₂ is adjusted to provide an OD₂₄₀ of 0.52 to 0.55. Solution A (0.1 ml) is added to Solution B (2.9 ml), and the time in minutes for the OD₂₄₀ to decrease from 0.45 to 0.40 is measured. This corresponds to the degradation of 3.45 μmoles H₂O₂. Therefore the specific activity (SA) can be calculated:

$$SA = (3.45 \div T) \times P \quad \text{Umg}^{-1} \quad \text{Where P} \quad = \quad \text{protein concentration} \\ = \quad 1 \div 15 \text{ mg/ml}$$

3.5.8 Assay for peroxidase activity

This was used to test for the presence of peroxidase activity in the presence of cofactors in the liquid extracts made from agar cultures (Section 3.7.2). The test for catalase activity could not be used because of interference at 240 nm by constituents of the liquid extracts.

The test enzyme was added to extract (5 mg/ml), to which was also added 20 μl of a 1% solution of H₂O₂. The mixture was incubated (37°C, 30 minutes) and assessed for the presence of any remaining peroxide (Section 3.9.6).

3.6 Serial culture of test LAB and indicator yeast.

3.6.1 Serial culture after growth of LAB to a fixed absorbance.

A starter culture of the test LAB was prepared, and 1 ml inoculated into prewarmed SGM (500 ml) in a screw top bottle. It was incubated with shaking at 30°C until a fixed absorbance (OD₆₀₀) was reached (absorbencies of 0.1, 0.3, 0.6 and 1.0 were the aim).

The culture was then centrifuged (8,000g, 4°C, 25 min) and the pH adjusted to 5.5 before syringe ultrafiltration (0.45 µm). The resulting sterile supernatant was stored at 4°C overnight. After warming to 30°C, 200 ml aliquots were dispensed into baffled conical flasks (500 ml capacity) and starter culture of *S. exiguus* was added (1 ml/l). 1 ml samples were removed at regular intervals for the measurement of yeast growth (as ΔOD₆₀₀).

3.6.2 Serial culture after growth of LAB for a fixed period of time.

A starter culture (Section 3.1.5) of the test LAB was prepared, and 1 ml inoculated into prewarmed SGM (500 ml) in a screw top bottle. It was incubated with shaking for a fixed time period (30°C, 120 rpm). The pH was adjusted to pH 5.5 before centrifugation (8,000g, 4°C, 25 min). The supernatant was warmed to 30°C, 200 ml aliquots were dispensed into baffled conical flasks (500 ml capacity), and a starter culture of *S. exiguus* was added (1 ml/l). Cultures were incubated with shaking (120 rpm, 30°C), and 1 ml samples were removed at regular intervals for the measurement of yeast growth (as ΔOD₆₀₀).

3.7 Extraction of antifungal activity from culture

3.7.1 Production of a cell free supernatant from liquid culture.

A starter culture (Section 3.1.5) of the test strain was inoculated into prewarmed SGM (0.1 ml in 200 ml SGM), and incubated with shaking (30°C, 120 rpm). The supernatant was obtained by centrifugation of the cultures (4°C, 10 min, 8,000 g) and filter sterilisation (0.2 µm).

3.7.2 Extraction of liquid from agar

Petri dishes containing the test bacteria were prepared as described in the 1 stage agar overlay assay (Section 3.5.2), except that the second (SSM) layer contained no indicator organism. The aerobic exposure period was 24 hours. The agar from these dishes was partially homogenised in a blender before being centrifuged (8,000g, 4°C, 60 minutes). The liquid obtained was filtered (Whatman N°1) to remove any remaining macroscopic agar particles. Using this method, approximately 650 ml may be extracted from 1 kg agar. Where an acidic extract was desired, the agar was fully homogenised in the presence of 2M HCl (120 ml/l agar). Full homogenisation was necessary to ensure that pH measured at the probe tip was an accurate reflection of the pH of the bulk of the preparation. The pH was adjusted to pH 1.9–2.0 by the further addition of 2 M HCl (up to around 45 ml/l agar).

3.7.3 Solvent extraction using immiscible solvents

Petri dishes containing the test bacteria were prepared as described in the 1 stage agar overlay assay (Section 3.5.2), except that the second (SSM) layer contained no indicator organism. The aerobic exposure period was 24 hours. The agar from 30 dishes (approximately 900 g) was homogenised in the presence of the desired solvent (300 ml). The homogenate was allowed to equilibrate for 5 hours at 4°C. Separation was achieved by centrifugation (10 minutes, 8,000 g). The upper organic phase was removed by

pipette and concentrated on a rotary evaporator. The maximum vacuum obtainable was used, and the temperature set at a point just below the boiling point of the mixture. If the boiling point was below room temperature, then the vacuum was reduced. The process was continuously monitored, and the temperature and vacuum adjusted as necessary. Where butanol was used, removal of all the solvent was not feasible, and the residue generally had a sludge like consistency. For bioassay by well diffusion (Section 3.5.3), the residue was suspended in water (10 ml) and the pH adjusted to 5.

3.7.4 Solvent extraction using miscible solvents

The method was essentially the same as that used for immiscible solvents (Section 3.7.3), except that the agar was first freeze dried, and the plates were extracted with a greater volume of solvent (2 extractions with 500 ml methanol each). Finally, the residue from the rotary evaporator was thoroughly dried in a freeze drier. For bioassay by well diffusion (Section 3.5.3), the residue was suspended in water (10 ml) and the pH adjusted to 5.

3.7.5 Acid precipitation

A liquid extract of agar (600 ml, Section 3.7.2) was prepared and the pH adjusted to 2 with concentrated (10 M) HCl. The precipitate was removed by centrifugation (8,000g, 4°C, 30 minutes), and resuspended in water. The pH was adjusted to 5 before bioassay by a well diffusion test (Section 3.5.3).

3.7.6 Ammonium sulphate precipitation.

A liquid extract of agar (600 ml, Section 3.7.2) was prepared and chilled to 4°C in a covered vessel. Ammonium sulphate (367 g) was added slowly with stirring, over a period of around two hours, to give a 90% saturated solution (Harris, 1989). The precipitate was removed by centrifugation (8,000g, 4°C, 30 minutes), and resuspended in phosphate buffer (0.01 M, pH 5). The resulting solution was bioassayed by a well diffusion test.

3.7.7 Size fractionation by dialysis

The dialysis tube was filled with agar extract (Section 3.7.2), sealed, and then placed in at least 10 volumes of either water or 30% PEG (MW 20,000). This was kept at 4°C with constant stirring for 48 hours. In the case of dialysis against water, the water was replaced after 24 hours, and the retentate concentrated by freeze drying. In both cases, the activity was assessed by bioassay in a well diffusion test (Section 3.5.3).

3.7.8 Size fractionation by ultrafiltration

A liquid agar extract (Section 3.7.2) was placed into the filtration unit and subjected to a pressure of around 2 kg/cm² at 4°C with constant stirring. The smallest volume that could be retained was dictated by the height of the stirrer bar. If the liquid level dropped below this, frothing occurred. For the large filtration unit, this volume was 40 ml (of a total capacity about 400 ml). For the small one, the volume was 20 ml (of a total capacity about 90 ml).

Where filtration was through a 500 or 1,000 MWCO filter, the concentration was three-fold – from an initial volume of 120 ml for the large filtration unit, and 60 ml for the small. Where filtration was through a 10,000 MWCO filter, the faster throughput meant that a ten-fold concentration could be achieved. For the small unit, this meant topping up the level with additional extract throughout the course of the filtration.

Where a serial filtration was used, the desired volume was passed through a 10,000 MWCO filter and collected in a covered sterile vessel. It was then filtered through a 1,000 MWCO filter.

The pH was adjusted to 5 before bioassay by a well diffusion test (Section 3.5.3).

3.8 Production of constitutive peroxide producing mutants of MTD/1

3.8.1 Development of an assay for glucose repressed peroxide production

This assay is based on the work of Berthier (1993). Basic Medium was prepared and supplemented, after autoclaving, with horseradish peroxidase (HRP, 3 or 5 mg/l) and tetramethyl benzidine (TMB, 250 or 500 mg/l), and some of the following (the precise combinations used are given with the results in Table 9.2):

Glucose,	0.2, 1, 5, 20 or 20 g/l
Fructose,	1, 5, 10 or 20 g/l
Galactose,	5 g/l
2-Deoxyglucose,	1 or 2.5 g/l
Citric acid,	2 g/l
Lactic acid,	2 g/l

Petri dishes contained 20 ml of the desired medium. They were spread with 100 µl of a 10⁻⁶ dilution of a starter culture of the desired organism, providing around 100 CFU per dish, and placed in anaerobic jars together with silica gel (40 g per gas producing sachet). The silica gel is necessary because the high moisture conditions provided by the anaerobic system would otherwise preclude discrete colony formation.

After 48 hours incubation (30°C), the plates were removed, exposed to a sterile airstream, and then returned to the incubator where they were left, unstacked, for up to 96 hours. In the presence of peroxide, TMB is oxidised by HRP to a blue compound, and so any changes in colour of either the colonies or the area immediately around them was noted.

3.8.2 Determination of caffeine sensitivity of *Lactobacillus plantarum* MTD/1

The cells from 1 ml of a starter culture (Section 3.1.5) were harvested by centrifugation. They were resuspended in mutant recovery medium (MRM, 1 ml) without HRP or

TMB, and with the addition of 2, 3, 4, 5 or 6 mg/ml caffeine. The incubation period was for 20 minutes at 37°C, after which a CFU count was made (Section 3.1.2).

3.8.3 Determination of EMS sensitivity of *Lactobacillus plantarum* MTD/1

MRM containing 6 mg/ml caffeine, but no HRP or TMB, was prepared. The cells from 1 ml of a starter culture (Section 3.1.5) were harvested by centrifugation. They were resuspended in 1 ml of the MRM, with the addition of 0, 5, 10, 20, 30, 50, 60, 70, 80, 90 or 100 µl EMS. The incubation period was for 10 or 20 minutes at 37°C, after which a CFU count was made (3.1.2).

3.8.4 Production of non-glucose repressed peroxide producing mutant of *Lactobacillus plantarum* MTD/1.

The cells from 1 ml of a starter culture (Section 3.1.5) were harvested by centrifugation. They were resuspended in 1 ml of MRM, with no HRP or TMB, but with the addition of caffeine (6 mg/ml) and EMS (70 µl). This cell suspension was incubated for 10 minutes in a water bath (37°C), after which 50µl of a 10⁻⁴ dilution was spread over Petri dishes of MRM (20 ml). The Petri dishes were placed in anaerobic jars together with silica gel (40 g per gas producing sachet, used to reduce humidity which would otherwise inhibit discrete colony formation).

After 48 hours incubation (30°C), the plates were removed, exposed to a sterile airstream and then returned to the incubator where they were left, unstacked, for 24 hours. Any colonies which were positive for peroxide production (a partial or full colour change from blue to white) were subcultured onto MRM plates and incubated anaerobically (48 hours, 30°C), and exposed once more to a sterile airflow. Single positive colonies from these subcultures were inoculated into MRS (10 ml) and incubated for 24 hours, before being stored as frozen cultures (Section 3.1.4).

3.9 Characterisation of mutant strains

3.9.1 Estimation of acetate concentration by HPLC

Culture supernatants were obtained by centrifugation and syringe filtration (0.2 μm). Analysis was done on a Biorad HPX-87H column designed for the separation of organic acids, set at 40°C. The mobile phase was H_2SO_4 (0.01468M), with a flow rate of 0.6 ml/min and a pressure of 2117 psi. Separated components were detected as modulations in the refractive index (RI 132 detector, set to a sensitivity of 0.15). The injection volume was 20 μl . Total run time was 30 minutes, with the acetate peak occurring at about 17 minutes.

The acetate peak was identified and integrated by the software, and the acetate concentration estimated by comparison with a standard curve. Standards consisted of serial two-fold dilutions of a known acetate sample (1.5 % down to 1.5/16 %, 1.5% was the maximum detectable concentration).

3.9.2 Preparation of a cell free extract

A starter culture (Section 3.1.5) of the required organism was prepared, and 1 ml inoculated into SGM (1 l) which was incubated, with shaking, overnight (120 rpm, 30°C). The cells were removed by centrifugation (15 minutes, 8,000 g, 4°C), and the cell pellet (~5 g) resuspended in sodium phosphate buffer (5 ml, 0.01 M, pH 7.0) in a 1.5 cm diameter Pyrex boiling tube. Glass beads (5 g, 250 μm) were added, and the mixture cooled on ice before vortexing (2 \times 90 second bursts, interspersed by cooling on ice for 180 seconds). The disrupted cell suspension was left to settle (on ice, 5 minutes) and the supernatant decanted into a Beckman 40 ml centrifuge tube. It was then centrifuged (10 minutes, 6,750 g, room temperature) and the supernatant decanted and filtered (0.2 μm). This cell free extract was kept on ice for immediate use, or frozen for later use (0.5 ml aliquots in screw-topped plastic tubes at -20°C). When required, samples were defrosted in a water bath (4°C) and then kept on ice.

3.9.3 Assay for total protein content

Total protein was assessed using a Sigma kit (690-A), which is a modification of the Lowry method. The recommended protocol was followed explicitly.

The reagents used were sodium chloride solution (0.85% w/v), Biuret reagent and Folin & Ciocalteu's reagent. The sample was diluted with sodium chloride solution until a final protein concentration of between 150 and 1,000 µg/ml was reached. Biuret reagent (2.2 ml) was added to the sample (0.2 ml), and the mixture allowed to stand at room temperature (10 minutes). Folin & Ciocalteu's reagent (0.1 ml) was added, and the mixture allowed to stand for a further 30 minutes. The OD₇₂₅ was measured, and protein content estimated by comparison with an albumin standard.

3.9.4 SDS-PAGE

The protein sample was added to an equal volume of SDS loading buffer and heated to 100°C for five minutes. Polyacrylamide gels were prepared with a 4% stacking gel and a 10% resolving gel, both were 3.3% C. The gel was loaded with approximately 25 mg of protein (100 µl of prepared sample). To allow an estimation of protein size, a marker, (kaleidoscope prestained standards, BioRad), was loaded at 20 µl per lane. This marker allows the size estimation before staining, but provides relatively broad bands so making the estimation procedure difficult. The electrophoresis cell was loaded into the gel tank which was filled with electrophoresis buffer. The electrophoresis was run at 20 mA through the stacking gel and 30 mA through the resolving gel until the dye front had reached the end of the gel.

The gel was stained by shaking gently, for 1–2 hours, in sufficient Coomassie blue to cover the gel. Excess stain was removed by soaking overnight in a large volume of Coomassie blue destain. The gel was then photographed and stored in a sealed bag containing 20% glycerol in water.

3.9.5 Assay for NADH oxidase activity

This method is based on that given by Anders (1970).

Stock solutions were made of β -NADH (0.943 mg/ml) and FAD disodium salt (0.688 mg/ml) in phosphate buffer (0.037 M, pH 7.0). The assay contained:

β -NADH solution:	120 μ l	8.3 μ M
FAD solution:	120 μ l	0.133 mM
Phosphate Buffer:	950 μ l	0.037 M, pH 7.0
Cell free extract:	10 μ l	

The constituents apart from cell free extract were mixed, and the extract was added and mixed to start the reaction. The reaction was followed by recording the OD₃₄₀ from 30 to 150 seconds using a Camspec M350 spectrophotometer, which calculated the Δ ODmin⁻¹ by linear regression. Since the molar extinction coefficient (ϵ) of β -NADH at 600 nm is $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, it can be shown that the NADH oxidase activity, A , is:

$$A = 23.15 \times (\Delta\text{ODmin}^{-1}) \quad \mu\text{M/ml extract}$$

3.9.6 Assay for peroxides

This was a colorimetric assay which was based upon the chromogenic oxidation of TMB by peroxides in the presence of Horse Radish Peroxidase (HRP). It was used to determine the peroxide content of the mutant strain cultures, and also in the estimation of peroxidase activity in extracts of the antifungal producing strains (Section 3.5.8).

Two solutions were prepared in buffer (0.01 M PO₄, pH 5). Solution *A* contained HRP (10 mg/ml), solution *B* contained TMB (6.7 mg/ml). The assay was initiated by adding *A* (50 μ l) and *B* (50 μ l) to the test sample (900 μ l). After 6 minutes at room temperature, the OD₄₅₀ was read. The amount of peroxide present may be estimated by comparison with H₂O₂ standards (up to 1 mM). Quantitative estimation was not possible where culture extracts or supernatants were used, because the addition of TMB caused a

precipitate which interfered with the OD measurement. Qualitative estimations were possible (by visual inspection of the presence or absence of blue pigment).

3.10 Laboratory-scale silo method for observing aerobic spoilage.

This work was conducted at the laboratories of Ecosyl Products Ltd.

Starter cultures of the test organisms were prepared by taking a single colony from a stock culture on MRS agar and inoculating into MRS (50 ml). This culture was incubated with shaking (30 hours, 30°C). An aliquot (0.1 ml) was added to fresh MRS (50 ml) and incubated with shaking (19 hours, 30°C). The CFU/ml in these starter cultures was estimated (Section 3.1.2).

An aliquot, calculated to be sufficient to provide 1×10^6 CFU per kilogram of maize to be treated, was taken from a starter culture. To this aliquot was added sufficient ¼-strength Ringers solution to provide 10 ml of inoculant per kg maize.

The maize used was stored frozen, and defrosted overnight prior to use. Maize silage that had previously undergone aerobic spoilage was added to this, to ensure that there were enough spoilage organisms to initiate aerobic spoilage in the control silos. This mouldy-maize silage was added to the defrosted maize (1 g/kg), and the whole was mixed thoroughly.

The maize was divided into lots, one per treatment group, and each lot was inoculated with the previously prepared cultures using a spray gun (10 ml/kg maize). Inoculation was carried out in a fume hood, to prevent cross-contamination of the samples. The samples being inoculated were repeatedly mixed during the procedure, to ensure an even dosage.

A sample was removed for microbiological analysis (Section 3.1.1). The remainder was used to provide four 2.5 kg portions, which were packed into four laboratory-scale silos (Figure 3.1). These consisted of a rigid plastic tube into which the maize was firmly compressed, with a valve to prevent gaseous build up. The silos were weighed pre- and post-packing, and the total ensiled material calculated. They were then placed at ambient temperature for 68 days.

At the end of the ensilage period the silos were weighed again, this enabled losses during the ensilage period to be calculated. They were then opened, and any visible signs of mould (which indicated incomplete sealing) was removed, along with the surrounding silage. The remaining contents were thoroughly mixed, and two samples removed, one for microbiological analysis (Section 3.1.1) and the other for chemical analysis. The sample for chemical analysis was frozen (-20°C) before being delivered to Jealott's Hill Laboratories who were responsible for the analysis.

A precisely-weighed sample of approximately 250 g was also removed from each silo for the aerobic spoilage study. Each 250 g sample was further mixed to ensure all aggregates had been disrupted, and then this loose preparation was placed into a plastic bag. The bags had been previously prepared by piercing them in four places, to enable airflow. The bags were placed into an insulating expanded-polystyrene box. The mouth of each bag was rolled down and left unclosed to expose the surface of the sample. The boxes were loosely closed with an expanded-polystyrene lid. A thermocouple was inserted through each lid into the centre of the sample, for temperature measurement. The thermocouples were connected to an electronic data logger which recorded the sample temperature at hourly intervals. The control temperature was read by two thermocouples, each of which were placed in a box prepared in a similar way to the sample, but with no maize silage. All the boxes were placed in a insulated room at ambient temperature.

The aerobic-spoilage period lasted 257 hours, after which samples were reweighed to allow the losses during the aerobic phase to be calculated. Portions were also removed for chemical and microbiological analysis, as above.

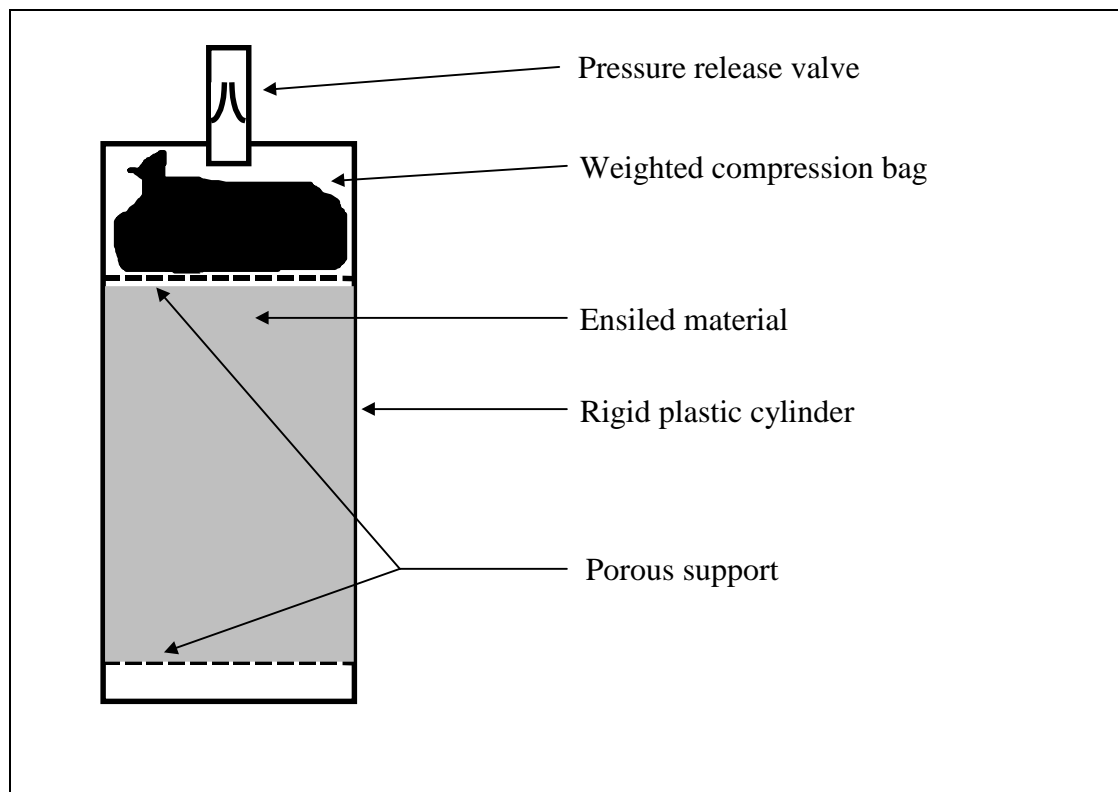


Figure 3.1. Schematic diagram of a laboratory-scale silo.

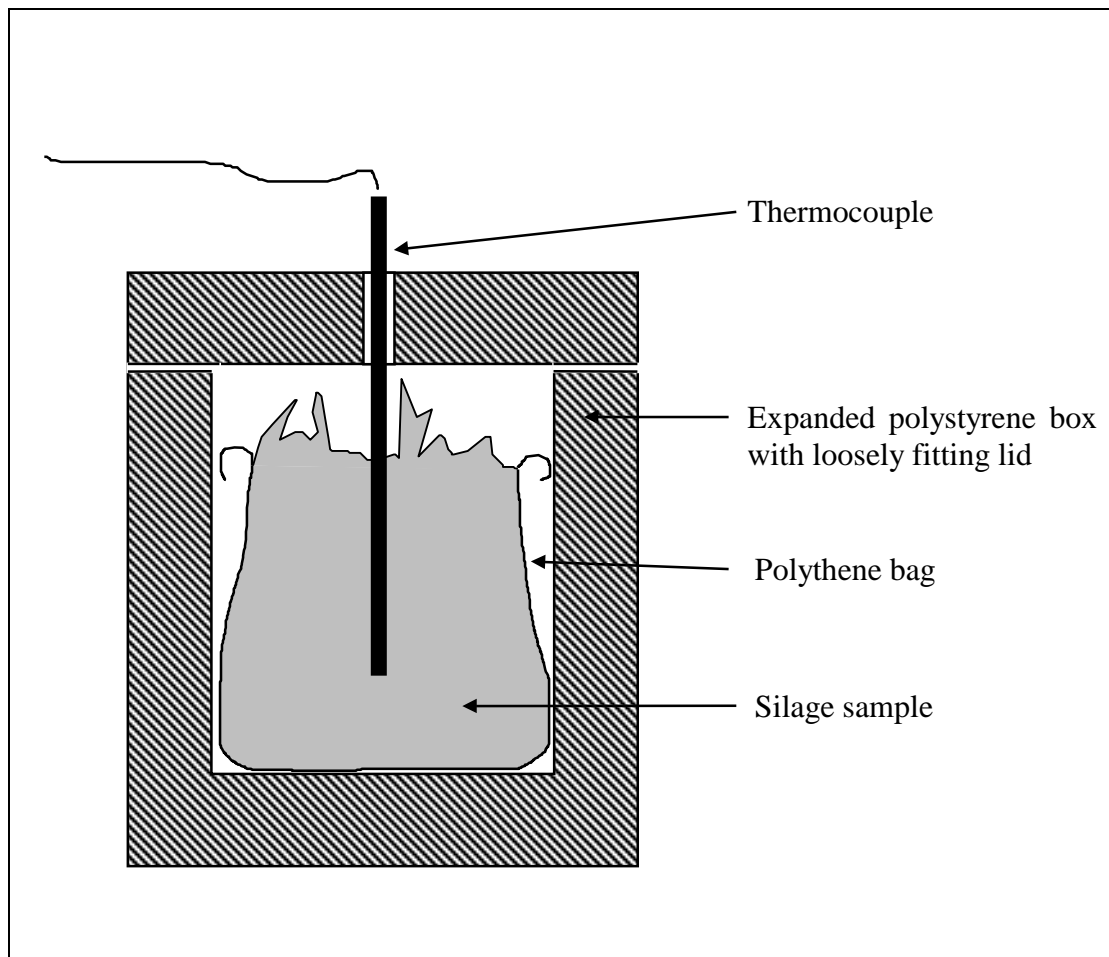


Figure 3.2. Schematic diagram of device for allowing aerobic spoilage of silage under controlled conditions.

3.11 A telephone survey of dairy farmers' attitudes towards the production of high dry matter silage

The target group for the survey was selected at random from a list of dairy farmers of England and Wales provided by the Milk Marketing Board. Although a regional analysis of the data was made, no attempt was made to obtain an equal sample size from each region since this would have skewed the overall data set. A total of 125 dairy farmers were interviewed over a period from the 28th March to 20th April 1995.

Some of the questions used in the survey were contingent on answers given to previous questions. The flowchart for the survey is given in Figure 3.3, and the actual form used is given in Figure 3.4. The opening questions are designed to find the amount of silage produced, awareness of HDM, and whether the farmer makes, or plans to make, HDM silage. Question 5 ("What are you planning to change to?"), was asked in order to discover what proportion of farmers would, unprompted, state an intention to make HDM silage.

Succeeding questions then ask what benefits and problems are perceived to be inherent to the production of HDM silage. These factors which dictate the decision to make HDM were identified by the respondent without prompting. Finally, information on the farmer's use of a contractor or an additive was obtained.

In order to facilitate analysis and allow the factors influencing HDM production to be isolated, the results were processed in a variety of ways and entered into a custom-designed database (built using Microsoft Access v2.0).

A regional analysis was made by allocating respondents to one of the regions (Wales, North West and Midlands, South West, North East and South East) on the basis of the county in which they were located.

The total tonnage, acreage or bales produced allowed categorisation into farm size (big or small). This was done by assuming that those farms producing more than 1000 t, or greater than 700 bales, or farming more than 60 acres for silage, were large farms.

Farms were also classified according whether the farm made, planned to make or did not make HDM silage, whether a contractor was used at any stage of the operation, and whether an additive of any kind was used. Some farmers stated that an additive was only used if the conditions were too wet to make HDM silage. These farmers were classified as additive users.

The factors identified by respondents as being important considerations in the decision to make HDM silage were superficially very varied, but it was possible to group them into underlying subject areas. A total of nine underlying subject areas were identified, and all the responses were categorised accordingly. The subject areas identified are listed below, along with all the responses which were assigned to them.

Better food: Assigned to responses of; better food, increased intake, more palatable, less acidic, better fermentation, better performance, healthier cows.

Decreased Effluent: Assigned to responses of; less effluent, less liquid.

Weather causes problems: Assigned to responses involving weather such as rain or general unpredictability

Spoils: Assigned to responses of; Heating at face, mould growth/mouldy silage, secondary fermentation (where clarified to mean aerobic spoilage), poor consolidation (where clarified to mean resultant aerobic spoilage).

Easier Handling: Assigned to responses of; easier handling, decreased bulk, fewer bales.

Other: Assigned to responses not otherwise categorised, including such things as; excessive dryness, picking up soil contamination, better for cow's feet, uses less straw to bulk,

no need for an additive, nitrogen levels not so critical, less acidic, better structure, cleaner cows, and high consumption meaning silage will not last as long.

Time consuming: Assigned to responses of; time consuming, and where labour costs or cost of machinery precludes manufacture.

Contractor won't handle: Assigned when contractor won't provide a HDM service, because of lack of machinery, scheduling difficulties or worries about damage to machinery.

Worse food: Responses are generally the same as for better food (above), but applied to low dry matter silage.

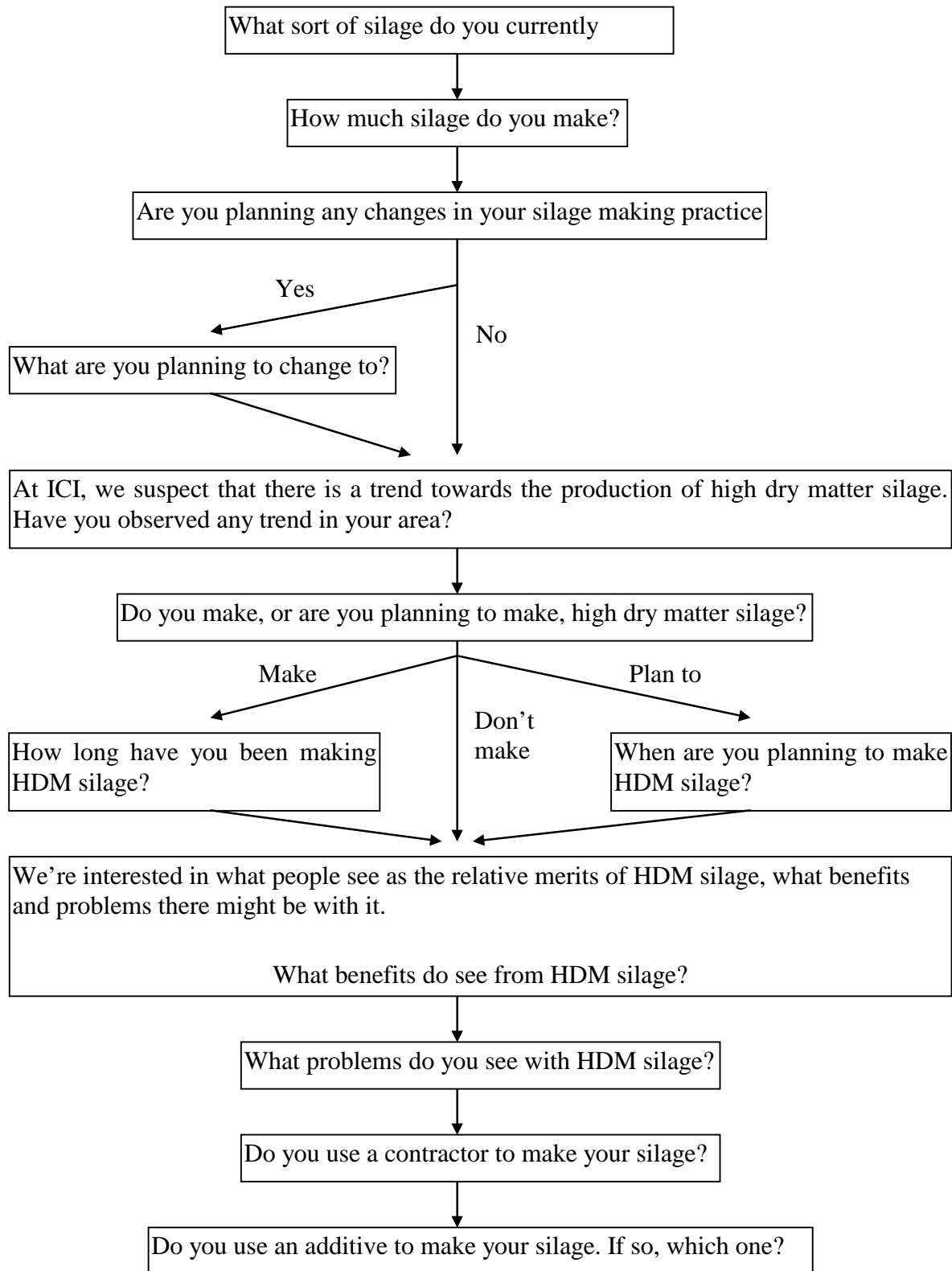


Figure 3.3. Flowchart for the phone survey of dairy farmers

The production of High Dry Matter silage
Survey Questionnaire

Name: _____

Location: _____

Phone Number: _____

Date: _____

(**Preamble:** Hello, I'm from ICI. We're conducting a survey of silage making practice around the country. Would it be possible for me to briefly ask your opinion on a few things?)

1. What sort of silage do you currently make?
 Clamp ____ Bale ____ Maize ____ (Go to 2)
2. How much silage do you make?
 Clamp ____ Bale ____ Maize ____
3. Are you planning any changes in your silage making practice?
 Yes ____ (Go to 4) No ____ (Go to 5)
4. What are you planning to change to?
 _____ (Go to 5)
5. At ICI, we suspect that there is a trend towards the production of high dry matter silage. Have you observed any trend in your area?
 Yes ____ No ____ (Go to 6)
6. Do you make, or are you planning to make, high dry matter silage?
 Plan to ____ (Go to 7) Make ____ (Go to 8)
 No plan ____ (Go to 9) Have made ____ (Go to 9)

Figure 3.4 (Continued next page). The questionnaire used for the phone survey of dairy farmers.

7. When are you planning to start making HDM silage? _____ (Go to 9)

8. How long have you been making HDM silage? _____ (Go to 9)

9. We're interested in what people see as the relative merits of high dry matter silage, what benefits and problems there might be with it.

What benefits do you perceive in HDM silage? _____
_____(Go to 10)

10. What problems do you see with HDM silage? _____
_____(Go to 11)

11. Do you use a contractor to make your silage?

Yes _____ No _____ (Go to 12)

12. Do you use an additive to make your silage. If so, which one?
_____(Go to 13)

13. (Reiterate main points made by interviewee)

Thank you for your help.

Figure 3.4 (Cont.). The questionnaire used for the phone survey of dairy farmers.