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Technologies for restricting mould growth on baled silage

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1. Introduction

Silage is made on approximately 86% of Irish farms, and 85% of these make some baled silage. Baled silage is particularly important as the primary silage making, storage and feeding system on many beef and smaller sized farms, but is also employed as a secondary system (often associated with facilitating grazing management during mid-summer) on many dairy and larger sized farms (O'Kiely *et al*., 2002).

Previous surveys on farms indicated that the extent of visible fungal growth on baled silage was sometimes quite large, and could be a cause for concern. Whereas some improvements could come from applying existing knowledge and technologies, the circumstances surrounding the making and storage of baled silage suggested that environmental conditions within the bale differed from those in conventional silos, and that further knowledge was required in order to arrive at a secure set of recommendations for baled silage systems.

This report deals with the final in a series (O'Kiely *et al*., 1999; O'Kiely *et al*., 2002) of three consecutive research projects investigating numerous aspect of the science and technology of baled silage. The success of each depended on extensive, integrated collaboration between the Teagasc research centres at Grange and Oak Park, and with University College Dublin. As the series progressed the multidisciplinary team needed to underpin the programme expanded, and this greatly improved the amount and detail of the research undertaken.

The major objective of the project recorded in this report was to develop technologies to improve the "hygienic value" of baled silage. Specifically, the stated aims were to:

1. Characterise the mycobiota on baled silage in Ireland
2. Enhance our understanding of the fermentation kinetics (and the unique combination of factors regulating them) peculiar to baled silage
3. Develop the capability to elucidate the mechanisms of gas entry to and exit from wrapped bales
4. Develop improved plastic and sealing methodologies
5. Identify strategies to successfully produce baled silage with a reduced content of mould and other undesirable micro-organisms.

2. Characterisation of the mycobiota of baled silage in Ireland

Specifically, the aims of this part of the project were:

1. Identify the types of fungus on baled silage on Irish farms, and quantify their incidence
2. Determine the response of the main fungi present to the micro-environmental (physical and biochemical) conditions likely within wrapped bales

Experiment 2.1: A note on sampling baled grass silage for fungal propagules

[M. O'Brien, O'Kiely, P., Forristal, P.D., Fuller, H.T.]

Prior to this study, there was no method described in the literature to sample baled grass silage for fungal propagules and information on the distribution of fungal propagules in individual silage bales was limited. It was not known if one cored sample per bale would accurately reflect the number and diversity of fungal propagules in the entire bale. The seemingly random appearance of visible mould colonies on bales suggested that fungal propagules may have a heterogeneous distribution within bales and therefore, more than one sample may need to be taken per bale. The objectives of the study were to determine the size and variability of fungal propagule populations, both within individual bales and between bales that were devoid of visible fungal growth.

Materials and methods: In February 2003, at Oak Park Research Centre, Carlow, ten cylindrical-shaped 'round' bales (1.2 m x 1.2 m, diam x length) of silage were chosen from a collection of 40 bales that had been harvested the previous summer from a single crop of perennial ryegrass and wilted for two days. The bales had been wrapped in four layers of black polythene stretch-film and stored outdoors on their curved sides for approximately eight months prior to sampling. Access to a collection of 40 bales ensured that at least 10 bales would be available that were free of both damage to the plastic stretch-film and visible mould or yeast growth. Individual bales were sampled at eight positions (considered the maximum number of samples that could be processed comfortably on a daily basis) with a sharpened cylindrical steel corer (length, 22 cm; inner and outer diameters 3.5 and 3.7 cm, respectively) powered by an electrical drill 1. Sampling points were at 2.00, 4.00, 8.00 and 10.00 h clock positions on each side of the bale barrel, about 40 cm from each end. At each sampling position, cores were taken to a depth of about 20 cm. Silage samples, each weighing between 60 and 70 g, were aseptically transferred from each core to clean plastic bags from which the air was then expelled and the bags sealed. The corer was disinfected between samples using 99 % alcohol. Mould and yeast counts were determined in duplicate 30 g sub-samples from each sampling position using the spread-plate technique and malt yeast extract sucrose agar (MYSA) as the enumeration medium. Mould and yeast propagules were differentiated based on their colonial features, and were enumerated separately as colony forming units (cfu) per gram wet weight of silage and expressed on a $\log_{10}$ scale. Dry matter (DM) concentration and pH were determined using silage samples (one 200 g grab sample per bale) that had no visible fungal growth present.

Results: The baled silage had 416 (S.D. 67.0) g DM/kg and a pH of 5.2 (S.D. 0.23). Both mould and yeast propagule numbers varied greatly between and within bales (Table 2.1.1). Yeast numbers differed significantly ($P<0.05$) both between bales and between core samples from within bales but the variability between bales (s.e. 1.44 $\log_{10}$ cfu/g) was greater than within the bales (s.e. 0.53 $\log_{10}$ cfu/g). The mould data were not amenable to analysis of variance due to the skewed distribution of values, with many values of zero.

Conclusions: The numbers of yeast and mould propagules can be relatively low in baled grass silage that is visibly free of mould growth. To overcome the effects of the heterogeneous distribution of fungal propagules in baled silage to be enumerated for moulds and yeasts, it is recommended that eight, or more, samples be taken per bale and these then composited to provide one representative silage sample for microbiological analysis.

Table 2.1.1 Yeast and mould colony forming units (cfu) in baled grass silage.

	Yeast[1], log_{10} cfu/g			Mould[2], log_{10} cfu/g		
Bale no.	Mean[3]	S.D.	Range	Mean[3]	S.D.	Range
1	<0.1	0.18	0-0.4	<0.1	0.09	0-0.3
2	2.6	1.23	0-3.6	<0.1	0.09	0-0.3
3	0.6	0.57	0-1.8	<0.1	0.09	0-0.3
4	2.3	0.64	1.2-2.8	0.1	0.20	0-0.5
5	3.1	0.57	2.0-3.8	0	0	0
6	2.8	1.84	0.3-5.1	1.4	2.23	0 - 6.3
7	3.4	1.22	1.2-4.7	1.1	1.97	0 - 5.7
8	2.9	1.77	1.1-6.0	1.0	1.73	0 - 5.2
9	1.8	1.75	0-4.5	1.2	1.76	0.3 - 5.4
10	2.6	1.49	0-4.5	0.5	0.87	0 - 4.7

[1]Yeast counts between bales and within bales were significantly different (P<0.05); [2]Mould counts were not subjected to analysis of variance as many bales had few or no moulds; [3]Mean of eight core samples per bale (mean of two sub-samples per core sample).

Experiment 2.2: Quantification and identification of fungal propagules in well-managed baled grass silage and in normal on-farm produced bales

[M. O'Brien, O'Kiely, P., Forristal, P.D., Fuller, H.T.]

In the present study, bales were sampled in order to quantify the 'background' fungal load that could potentially grow and contaminate silage should air gain access through the polythene film. The aim of the study was to determine the number and identity of the predominant viable mould and yeast propagules in parts of the outer horizon of bales where visible mould growth was not evident in well-managed bales (Experiment 2.2A) and in bales produced using normal on-farm procedures (Experiment 2.2B). It would be expected that all bales would have comparable populations of epiphytic microbes at the time of wrapping and that subsequent differences in microbial populations would reflect the consequences of different wrapping and storage of the bales.

Materials and methods: *Experiment 2.2A: Well-managed bales:* From July to August 2004, 15 cylindrical ('round') bales (1.2 x 1.2 m) of freshly harvested grass were obtained from eight commercial farms located within a 10 km radius of the Grange Beef Research Centre. One to two bales were collected from each farm and, in the case of two bales, each was sourced from a different field. Four different balers were used and the bales were tied with netting. Bales were transported to Grange where they were weighed and wrapped (McHale™ 991 BE, Mayo, Ireland) in six layers of black polythene stretch-film (Volac Silawrap®, Co. Wexford, Ireland). The time interval between baling and wrapping was less than two hours. Bales were stored on their curved sides on a sand base, in a protected area. A questionnaire was completed relating to the history of each crop – the duration of wilting, type and age of the grass crop and weather conditions from cutting to baling. Information on grass chopping and additive-use practices was also obtained. After six weeks storage, these bales were examined as in Experiment 2.1. Each bale was sampled at eight locations using a sharpened cylindrical steel corer (length, 22 cm; inner diameter, 3.5 cm) powered by an electric drill. The corer was disinfected between bales using 95 % industrial methylated spirits. Sampling locations were at 2.00, 4.00, 8.00 and 10.00 h clock positions on the bale barrel, about 40 cm from each end and cores were taken to a depth of about 20 cm. The eight core samples from each bale were aseptically transferred to a clean plastic bag, the air expelled and the bag sealed. The 15 bale samples were stored at 4 ºC prior to microbiological analysis later in the day. The composited silage sample from each bale was thoroughly mixed under aseptic conditions and three 30 g sub-samples were removed for microbiological analyses.

Experiment 2.2B: On-farm produced bales: In February 2005, two cylindrical ('round') bales (1.2 x 1.2 m) of grass silage were examined on each of nine commercial farms. The farms were within a 7 km radius of Grange and did not include any of the farms in Experiment 2.2A. The two bales selected on each farm were in readiness for feeding to livestock and were from the same grass cutting, while crop composition differed among farms. All 18 bales had been harvested the previous summer (i.e. 2004) and were in storage for approximately eight months. Any damage to the film surrounding the bales, and visible mould growth on the bale surfaces, was noted at the time of sampling. A questionnaire was completed on each farm visited, with information being

sought from the farmer on harvesting and bale management practices. Bales were sampled and microbiological analyses completed as in Experiment 2.2A. However, sample cores were collected from locations on bales as close as possible to the intended sampling points (as described in Experiment 2.2A), taking particular care to avoid visibly mouldy silage, if present. Moulds and yeasts were enumerated and identified as described above.

Results: *Experiment 2.2A: Well-managed bales:* The 15 bales used in Experiment 2.2A reflected the diversity of baled grass silage used on Irish farms (Table 2.2.1). The farmer questionnaires revealed that bales were made from grass harvested from new and old permanent pastures, foliage was both leafy and stemmy, weather conditions between mowing and baling varied from fine to rainy and the duration of wilting ranged from 3 - 72 h. Typically, grass was sliced by stationary knives on the baler at baling, no additives were applied and bales were wrapped with netting. There was no visible damage to the polythene film on any bale following the six-week storage period and all bales were free of visible mould growth when the polythene film was removed.

Yeasts were found in core samples from all bales, whereas moulds were detected in samples from 9/15 bales. The mean yeast cell number for the 15 bales was 9.7×10^3 cfu/g silage, ranging between $<10^1$ to 10^5 cfu/g, while for moulds there were $<10^1$ cfu/g. Ten species of yeast were cultured, identified and enumerated from these bales (Table 2.2.2). Some yeast species occurred in higher numbers than others, for example, *Saccharomyces exiguus* (anamorph, *Candida holmii)* numbers ranged from 0 to 10^5 cfu/g whereas *Pichia fermentans* ranged from 0 to 10^3 cfu/g. *S. exiguus,* cultured from 12 bales, was the predominant yeast in this group of bales. *P. fermentans* and *Candida glabrata* (synonym, *Torulopsis glabrata)* were isolated from eight and five of the 15 bales, respectively. Mould propagules were not as common as yeast cells in bale samples and, when they did occur, numbers did not exceed 10^2 cfu/g. *Cladosporium, Byssochlamys* and *Penicillium* species were isolated from a small number of bales in very low numbers. Other moulds cultured were not identified further because of low numbers enumerated and their infrequent occurrence as bale inhabitants. Correlation coefficients of the bale characteristics and their level of significance are in Table 2.2.3. Both forage DM and pH were positively correlated to increasing mould numbers. There was no correlation between either forage DM or pH and yeast numbers.

Experiment 2.2B: On-farm produced bales: As in Experiment 2.2A, the bales (n=1 8) used in this experiment were equally diverse in grass composition and chemical analysis (Table 2.2.1). Farmer questionnaires indicated that they were from May to September 2004 cuts, from grass cut in both recently seeded and older pastures, that the foliage was mostly stemmy, that the weather between mowing and baling was dry and that wilting was carried out for 24 h, on average. Grass was sliced at baling, no additives were used and bales wrapped in netting were covered in four layers of black polythene stretch-film. Damage was observed in the polythene film on 8/18 bales after eight months storage. The silage in bales with damaged film was invariably contaminated with visible moulds, but equally the film on a small number of bales was not visibly perforated but moulds were still evident when the polythene was removed. Proportionally, 0.8 (15 of 18) bales were contaminated with visible surface mould growth that was identified *in situ* as predominantly *Penicillium.*Numbers and types of fungal propagules varied among bales. Yeasts were cultured from all bale samples and moulds were cultured from 15/18 bales. Mean yeast numbers in the 18 bales were higher than mould numbers (2.3×10^5 and 1.5×10^5 cfu/g, respectively). Bales with visible damage to their polythene film (n = 8 bales) had higher mean numbers of yeast and mould (3.2×10^5 yeast cfu/g and 2.7×10^5 mould cfu/g) than bales (n = 10) where the film appeared intact (1.5×10^5 yeast cfu/g and 5.7×10^4 mould cfu/g). The higher numbers of mould propagules in bales with damaged film differed ($P<0.05$) from bales with undamaged film, but there was no difference in yeast numbers. Mean numbers of yeast cells and mould propagules in bales contaminated with visible surface mould growth (n = 15 bales) were higher (2.8×10^5 yeast cfu/g and 1.8×10^5 mould cfu/g) than in bales (n = 3) free of visible surface mould growth (1×10^2 yeast cfu/g and $<10^1$ mould cfu/g). Differences in yeast and mould numbers among bales from the same farm may reflect different levels of polythene film damage to individual bales. Of seven yeast species identified (Table 2.2.2.), *Pichia fermentans* was the most common (11/18 bales) and its cell numbers in the bales ranged from 0 to 1.5×10^6 cfu/g (mean, 1.2×10^5 cfu/g). *Saccharomyces exiguus* and *Pichia anomala* were isolated from six and five of the 18 bales, respectively. The identification of yeasts using an ID32C identification kit and additional physiological tests proved satisfactory in most cases. It was observed, however, that 34 putative *S. exiguus* isolates cultured from bales in both experiments varied greatly in their vitamin requirements. For example, 5/34 isolates did not require any vitamins for growth, 22/34 required only biotin, 2/34 required both biotin and pantothenate, and 5/34 required vitamins other than biotin and pantothenate.

Penicillium roqueforti Thom was the predominant mould, cultured from 13/18 bale samples and viable propagule numbers in silage ranged from 0 to 7.1×10^5 cfu/g (mean, 1×10^5 cfu/g).

Mucoraceous moulds (3/18 bales) and *Trichoderma*(3/18 bales) were the next most frequently isolated moulds. Correlation coefficients of the different bale characteristics and their levels of significance are in Table 2.2.3. The only significant correlation was between increasing mould numbers and increasing yeast numbers.

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Conclusions: Good management can dramatically reduce the numbers and types of yeast and mould propagules in baled grass silage. Bales produced using normal on-farm procedures had higher mould counts (primarily of *P. roqueforti)* most likely because the integrity of the polythene film surrounding these bales was compromised during the storage period. When mould colonies were visibly present on baled silage, other parts of the bales were likely to have propagule counts that were raised, albeit to a lesser extent than published for visually mouldy silages. It is not clear from this study if the propagules were spores or actively growing mycelia from adjacent spreading mould colonies. This study has shown that a substantial fungal spora can be found in baled silage and that in order to prevent such propagules germinating and growing, a high standard of bale wrapping, handling and storage is required to ensure maintenance of an anaerobic environment within bales.

Table 2.2.1 Chemical composition of baled grass silage used in Experiments 2.2A and 2.2B.

	Experiment 2.2A[1]		Experiment 2.2B[2]	
	Mean	S.D.	Mean	S.D.
Bale weight (kg)	718	141.2	nd	nd
Dry matter(DM)(g/kg)	310	92.0	303	58.1
Ash (g/kgDM)	96	15.4	94	13.1
in vitro DM digestibility (DMD) (g/kg)	644	75.4	684	68.8
Crude protein (g/kgDM)	127	26.0	145	31.4
Ammonia-nitrogen (N) (g/kgN)[3]	52	24.1	79	42.0
pH	4.2	0.54	4.4	0.17
Fermentation products [3, 4]				
Ethanol (g/kgDM)	3.0	0.82	6.5	3.99
Lactic acid (g/kgDM)	56.6	27.79	56.6	15.4
Acetic acid (g/kgDM)	5.0	1.37	7.2	2.46
Propionic acid (g/kgDM)	0.9	0.28	1.2	0.73
Butyric acid (g/kgDM)	3.4	2.60	4.5	3.47
Total fermentation products (g/kgDM)	68.9	26.16	76.0	18.67
Lactic acid/fermentation products (g/g)	0.78	0.12	0.74	0.05

nd, not determined; [1] Fifteen bales of grass silage representing 15 diverse crops prepared and stored under well-managed farm conditions; [2] Eighteen bales of grass silage obtained from nine farms, prepared and stored using normal on-farm procedures; [3]Ammonia-N and fermentation product data was only available for 9/15 bales in Experiment 2.2A; [4]Fermentation products: Lactic, acetic, propionic and butyric acids and ethanol.

Table 2.2.2 Comparison of fungal taxa and their propagule abundance (cfu/g silage) in core samples from well-managed bales (Experiment 2.2A) and normal on-farm produced bales (Experiment 2.2B).

Fungal genera/species	Experiment 2.2A (n=1 5 bales)			Experiment 2.2B (n=1 8		
	No. of bales in	[1]Mean cfu/g	[3]Maximum cfu/g	No. of bales in	[2]Mean cfu/g	[4]Maximum cfu/g
Yeasts:						
Saccharomyces	12	8.4×10^3	1×10^5	6	2.7×10^4	4.2×10^5
Pichiafermentans	8	$<10^2$	1×10^3	11	1.2×10^5	1.5×10^6
Candida glabrata	5	3.6×10^2	4.1×10^3	1	$<10^1$	10^1
Saccharomyces	2	$<10^1$	$<10^2$	0	0	0
Torulaspora	2	$<10^1$	$<10^2$	0	0	0
Issatchenkia	2	$<10^1$	10^2	1	7×10^4	7×10^4
Pichia anomala	1	$<10^2$	$<10^2$	5	1.6×10^4	2.7×10^5
Candida rugosa	1	$<10^1$	$<10^1$	0	0	0
Kluyveromyces	1	$<10^1$	$<10^1$	0	0	0
Geotrichum spp.	1	$<10^1$	$<10^1$	3	2.6×10^2	2.5×10^3
Debaryomyces	0	0	0	1	$<10^1$	$<10^1$
Other yeasts [5]	3	8.9×10^2	1.3×10^4	0	0	0

[1] Mean numbers of fungal propagules in 15 bales; [2]Mean numbers of fungal propagules in 18 bales; [3]Maximum numbers of fungal propagules in any of the 15 bales; [4]Maximum numbers of fungal propagules in any of the 18 bales; [5]A variety of other yeast and mould species present in low numbers.

Table 2.2.2 Continued over/

Table 2.2.2 continued Comparison of fungal taxa and their propagule abundance (cfu/g silage) in core samples from well-managed bales (Experiment 2.2A) and normal on-farm produced bales (Experiment 2.2B).

Fungal genera/species	Experiment 2.2A (n=1 5 bales)			Experiment 2.2B (n=1 8 bales)		
	No. of bales in which present	[1] Mean cfu/g	[3]Maximum cfu/g	No. of bales in which present	[2]Mean cfu/g	[4]Maximum cfu/g
Moulds:						
Penicillium roqueforti Thom	0	0	0	13	$1x10^5$	$7.1x10^5$
Penicillium paneum Frisvad	0	0	0	2	$4.6x10^4$	$8.3x10^5$
Other *Penicillium*	1	$<10^1$	$<10^1$	0	0	0
Trichoderma	0	0	0	3	$7.7x10^2$	$1.3x10^4$
Cladosporium	1	$<10^1$	$<10^2$	0	0	0
Byssochlamys	3	$<10^1$	$<10^1$	0	0	0
Mucoraceous mould	1	$<10^1$	$<10^1$	3	$7.6x10^2$	$1.3x10^4$
Other moulds[5]	9	$<10^1$	$<10^1$	6	$<10^2$	$1x10^3$

[1] Mean numbers of fungal propagules in 15 bales; [2]Mean numbers of fungal propagules in 18 bales; [3]Maximum numbers of fungal propagules in any of the 15 bales; [4] Maximum numbers of fungal propagules in any of the 18 bales; [5]A variety of other yeast and mould species present in low numbers.

Table 2.2.3 Correlation coefficient *(r)* values of baled silage characteristics.

Experiment 2.2A [1]	pH	Bale wt	Mould	Yeast
Dry matter	0.81 ***	-0.7 **	0.7 **	-0.39
pH	-	-0.71 **	0.55 *	-0.32
Bale wt	-	-	-0.75 ***	0.48 *
Mould	-	-	-	-0.52 *
Experiment 2.2B [2]				
Dry matter	0.14	na [3]	-0.06	-0.14
pH	-	na	0.03	-0.09
Bale wt	-	-	na	na
Mould	-	-	-	0.67 ***

* $P<0.05$; ** $P<0.01$; *** $P<0.001$. na, not applicable; [1] Fifteen bales of grass silage representing 15 diverse crops prepared and stored under well-managed farm conditions; [2] Eighteen bales of grass silage obtained from nine farms prepared and stored using normal on-farm procedures; [3] Bale weights were not determined in Experiment 2.2B.

Experiment 2.3: Fungi isolated from contaminated baled grass silage on farms in the Irish midlands

[M. O'Brien, O'Kiely, P., Forristal, P.D., Fuller, H.T.]

The objective of this preliminary study was to identify the visible fungal growths on the surface of baled silage and determined the extent of their occurrence on 35 farms in the Irish midlands. Bale collections on these farms were also surveyed to establish the extent of occurrence of the macrofungus *Schizophyllum commune*.

Previous studies in the literature that looked at the mycobiota of baled silage have enumerated and identified fungal propagules from cored samples. Although this method proved useful when sampling silage devoid of visible fungal growth, it may not accurately reflect what fungi are present in visibly mouldy silage. In this study, small fragments of fungal material were collected from visible colonies growing on the surface of bales and these fungal samples were transferred directly onto culture media for isolation and identification.

Materials and methods: In March 2003, baled grass silage was surveyed on 35 farms along a 150 km route (from N 85 63 to S 12 10 (Irish National Grid)) in the Irish midlands. The route was sub-divided into five sections and seven farms were surveyed per quintile. A detailed questionnaire 1 was completed on each farm visited, with information being sought on harvesting and bale management practices. One to three bales were examined in detail on each farm; the bales chosen were those in readiness for feeding. The number of bales examined on each farm represented the usual number of bales fed daily to livestock. The total number of bales sampled was 64. Prior to removing the plastic film surrounding each bale, it was examined carefully for visible holes/damage. On removal of the plastic film, all visible mould and yeast colonies on the bale surface were located, numbered, sampled and photographed 2. Mould colonies were defined as areas of visible mycelial growth and yeast colonies as a diffuse creamy growth on the surface of the bale. For yeasts and visibly sporulating mould colonies, a sterile moist cotton swab was gently touched off the fungal material and replaced into a sterile sealed container. For mould colonies that were not visibly sporulating, a small fragment of foliage colonised with fungal material was aseptically transferred to an individual sterile sealable container. The surface area of each colony was determined by placing a plastic grid (individual square size = 5 x 5 cm) over the colony and visually estimating its area. The percentage of the total surface area affected with fungal growth was then calculated for each bale. Bale collections on the farms were also assessed for the occurrence of the macrofungus *Schizophyllum commune*. The proportion of bales on each farm where *S commune* was visibly evident protruding through the plastic film and its location on the bale surface was recorded.

Dry matter (DM) and pH were determined using silage samples (four composited sub-samples per bale) that had no visible mould. The pH of silage contaminated with visible mould was also recorded on site on farms. The readings were taken from the centre of large fungal colonies on bales by inserting a pH probe (Schott Blueline 12 pH probe) to a depth of 5 – 6 cm into the fungal contaminated silage. The direct-plating method was used to isolate the mould and yeast samples. Foliage bearing fungal material was transferred aseptically to the surface of malt extract agar (MEA, Oxoid, Basingstoke, UK) and dicloran rose bengal chloramphenicol agar (DRBC, Oxoid). DRBC was used to allow the growth of slow-growing fungi by inhibiting the growth of rapid growers. The antibiotics, chlortetracycline (50 µg ml^{-1}, Sigma) and chloramphenicol (100 µg ml^{-1}, Sigma) were added to MEA to inhibit bacterial growth. DRBC contained 100 µg ml^{-1} chloramphenicol (Sigma). Four foliage fragments (<1 cm in length) from each sample were placed onto each of the two media at four equidistant points. Cotton swabs with adhering fungal material were gently touched against the surface of the two media (four points per plate). Plates were incubated for between 3 - 14 d at 25 ºC depending on the growth rate of the fungi isolated. Fungi were sub-cultured onto MEA (Oxoid), incubated for 5 - 10 d (depending on the fungal species) and stored at 4 °C for later identification. Fungi were identified to genera or species by their macro- and micromorphology features using appropriate identification keys. Yeasts were not identified further. As an additional confirmation tool for *Penicillium* isolates, a representative number were screened for their secondary metabolite profiles using HPLC-UV with LC-MS.

Results: The majority of the bales examined had been harvested in June and July 2002. Bales had a DM content of 286 (S.D. 93.9) g/kg and a pH of 4.3 (S.D. 0.56). The mean pH of fungal-contaminated silage was 6.5 (S.D. 1.28), which was significantly (P<0.001) less acidic than clean silage from the same bales. Visible fungal growth was present on 58/64 (91 %) bales examined. On average, there were six visible fungal colonies on each affected bale and this ranged from one to twelve colonies per bale. The extent of fungal growth on the bale surfaces ranged from 0.1 to 14.9 % coverage, with a mean coverage of 5.1 %. Most bales had an area in the range 0.01 to 0.2 m^2 covered with visible fungal growth (Figure 2.3.1). Fungi appeared to be able to colonize any part of the bale surface. The percentage of the bale area affected with fungal growth when bales were stored on their ends (n=20 bales) and on their curved side (n = 44 bales) was 5.4 (S.D. 4.7) % and

4.2 (S.D. 3.8) %, respectively. As this difference was not statistically significant ($P>0.05$), it indicates that the extent of fungal colonisation is not contingent on how a bale is stored on the ground.

The plastic film of 25/64 (40 %) bales examined was visibly damaged. This level of damage is probably an underestimation as there may be micro-damage that is not readily visible to the human eye. Damaged bales had proportionally higher fungal coverage (7.0 %, S.D. 4.0) than where the film appeared intact (4.0 %, S.D. 3.4) and this was statistically significant ($P<0.05$). While damage was observed on all parts of bales, the plastic film was mostly observed to be damaged on the bales curved side. Bird and cat damage accounted for 35 % and 17 % respectively of all damage. Farm machinery, livestock and rodents caused other damage. Only 4 % of damaged bales were repaired and these were generally repaired using adhesive plastic repair patches. A total of 332 visible mould and yeast colonies were sampled on 58 bales, resulting in 444 fungal isolates. A single pure fungal culture was isolated from 209 samples taken from fungal colonies on bales. In the case of the other samples, two, three or even four different fungi grew from a single silage sample. On occasion, two fungi were observed co-existing with each other on the same part of the bale. In most cases the target fungus/fungi on the bale could be recognised in culture, isolated and identified. Occasionally fungi that were not obviously present at the time of sampling, grew out from silage samples in culture. These fungi may have arisen from dormant propagules in the silage. Ten fungal samples from bales failed to grow *in vitro,* representing 2.7 % of total isolates.

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Bale collections on the 35 farms were surveyed to establish the extent of occurrence of the macrofungus *S. commune.* This fungus was present in the form of a bracket mushroom visibly protruding through the plastic film on bales on 49 % (17/3 5) of the farms surveyed. When present, *Schizophyllum* was observed on less than 10 % of the bale collection on a farm; the curved sides and shoulders of bales were the areas most frequently affected.

Conclusions: This survey showed that fungal contamination of ruminant feed in the form of baled silage is common on farms in the Irish midlands. Although a relatively small number of fungal species was responsible for most of the contamination, at least two of these fungi *(P. roqueforti* and *P. paneum)* are capable of reducing silage quality and potentially causing health problems in livestock by their known ability to produce harmful mycotoxins. Air ingress to baled silage is a major factor in facilitating mould colonisation in a substratum otherwise inhibitory to fungal growth. Livestock owners who feed baled silage need to be aware of the potentially harmful effects that certain fungi pose to livestock and to employ effective measures to maintain the integrity of the plastic film in order to minimise fungal contamination on bales.

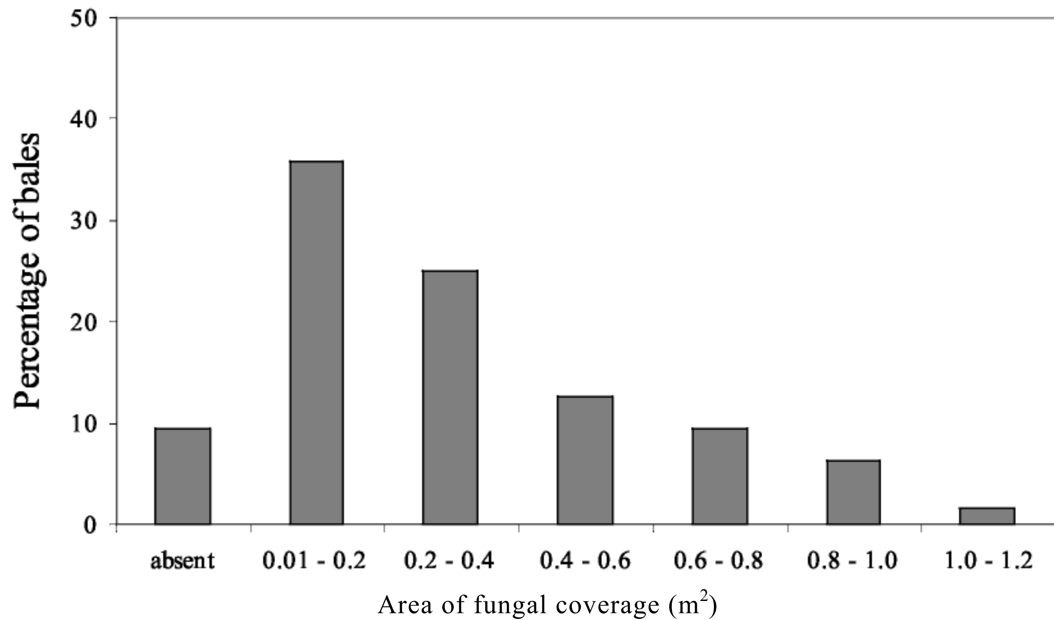


Figure 2.3.1 Extent of visible fungal growth on the surface of baled grass silage (n = 64) on a sample of Irish farms (n = 35). Fungal growth on bale surfaces was estimated using a plastic grid of a known area. Each bar represents the percentage of bales contaminated with fungal growth corresponding to the area range (m^2) of the bale surface affected with visible fungi. Total bale surface area = 6.78 m^2; 0.2 m^2 represents 3 % of bale surface area.

Table 2.3.1. Fungi isolated from contaminated baled grass silage on a sample of 35 Irish farms in Spring 2003.

Fungal genera/species	Number of isolates	Percentage of total isolates
Penicillium roqueforti	231	52.0
Penicillium paneum	20	4.5
Yeasts	60	13.5
Geotrichum	35	7.9
Mucoraceous spp.	27	6.1
Schizophyllum commune	19	4.3
Fusarium	5	1.1
Trichoderma	2	0.5
Coprinus	1	0.2
Other unidentified isolates	44	9.9
Total	**444**	

Experiment 2.4: Visible fungal growth on bale grass silage during the winter feeding season in Ireland and silage characteristics associated with the occurrence of fungi

[M. O'Brien, O'Kiely, P., Forristal, P.D., Fuller, H.T.]

In the preliminary survey (Experiment 2.3) it was reported that visible fungal contamination of bale grass silage was widespread on farms in the Irish midlands. That survey was undertaken at the end of the Irish feeding season in March, when bales were in storage for approximately nine months. In this more intensive survey of bales on midland farms, 20 bales on 10 farms were examined each month throughout the winter feeding period i.e. from November to March to determine the fungal species present on the bale surfaces and the extent of their establishment successively during the winter storage/feedout period. A further objective was to establish how colonisation by particular fungi related to silage characteristics and on-farm bale management practices.

Materials and methods: *Sample collection:* Between mid-November 2003 and mid-March 2004, baled silage was surveyed on 50 farms along a 165 km route (from latitude 53°59' N, longitude 07°21' W to latitude 52°40' N, longitude 07°49' W) in the Irish midlands. The route was sub-divided into quintiles with two different farms visited per quintile each month for five months (i.e. 10 different farms each month). The 50 farms encompassed different farm enterprises and geographical locations and were representative of a range of agricultural practices in this region. Farm visits took place on the following dates: 13 and 14 November, 11 and 12 December in 2003, 7 and 8 January, 19 and 20 February, and 18 and 19 March in 2004. A detailed questionnaire was completed on each farm visited, with information being sought from the farmer on numerous aspects of the baled silage-making process (Table 2.4.1); additional observations on bale storage practices were recorded on site by the authors (Table 2.4.2). The geographical location of bale collections was recorded using the Magellan GPS (Global Positioning System) Pioneer™ satellite navigator (San Dimas, CA).

Two bales in readiness for feeding were examined in detail on each farm (n=20 bales/month; total=100 bales). Bales, polythene wrapped, were cylindrical in shape with a nominal diameter and length of approximately 1.2 m. Most bales examined were stored on their flat end (0.7) rather than on their curved side (0.3); all bales were sourced from the ground tier. The examination entailed scanning the polythene film on each bale for damage and recording the extent, location and cause of damage (if identifiable). On removal of the polythene film, all visible mould and yeast colonies were located by lettered plastic marker and photographed. To determine the proportion of bale surface colonised by fungal growth, the visible surface area of each fungal colony was quantified using a plastic grid square of known area. The proportion of bale surface area affected by visible fungal growth was calculated per m^2 of the visible surface area observed which depended on bale orientation at the time of sampling. Surface area of an entire bale, visible surface area of a bale on its curved side and visible surface area of an upright bale are approximately 6.78, 5.76 and 5.65 m^2, respectively. The proportion of the bale surface area affected by visible fungal growth was calculated per m^2 of the visible surface area observed.

Every visible fungal colony was then sampled using protocols described earlier. Briefly, samples were collected either by touching a sporulating colony with a sterile moist cotton swab or by placing a small fragment of foliage colonised with fungal material into an individual sterile container that was then maintained at 4 °C until required. Samples of silage for chemical analysis were taken from parts of bales that were without visible fungal contamination or spoiled material. Four grab samples were taken respectively, at 2.00, 4.00, 8.00 and 10.00 h clock positions on the bale barrel surface and a fifth surface sample was taken randomly at one of the bale ends. All five sub-samples were composited in a clean polythene bag, stored at less than 4 °C during transport and then at –18 °C prior to chemical analysis.

A detailed questionnaire was completed on each farm visited, with information being sought from the farmer on numerous aspects of the baled silage-making process and additional observations on bale storage practices were recorded on site.

Results: In summer 2003, a mean of 224 bales/farm were harvested on the 50 farms included in the survey. Of these, means of 187, 109, 116, 112 and 67 bales/farm remained to be fed when farms were visited in successive months from November to March. The harvested herbage was from permanent temperate grassland of mixed botanical composition. Bales were stored for 14 to 41 weeks (mean and median = 28 weeks) before being fed to livestock. Farmers' answers to a questionnaire regarding production of baled silage are collated in Table 2.4.1 and the authors' observations on bale storage and polythene film damage are presented in Table 2.4.2. Both data sets and results of silage chemical analyses (Table 2.4.3) were used in RDA.

Visible fungal contamination of bales: Fungal growth was visibly present on 90 of the 100 bales examined (Table 2.4.4). The proportion of bales affected ranged from 0.75 (January) to 1.0 (March); of the 10 bales that had no visible fungal contamination, all were aged between 14 to 34

weeks on the day examined whereas all bales aged 35 to 41 weeks had visible fungal growth present. Bale age was calculated from the time bales were wrapped to when they were examined by the authors. On average, there were 6 visible fungal colonies on each affected bale and this ranged from 1 to 21 colonies per bale. The number of visible colonies on bales was higher ($P<0.05$) at the end of the feeding season (mean, 8; in March) than at the beginning (mean, 4, in November) (Table 2.4.4). While the youngest bales numerically had fewer fungal colonies present, there were no significant differences in colony numbers when compared with the oldest bales.

Surface areas colonised by fungi were numerically less on bales fed to livestock in November and on bales stored for the shortest time (14-20 weeks) relative to March and 35-41-week-old bales, respectively (Table 2.4.4).

Fungal spoilage and features of harvesting, baling, wrapping and storage: The number of bales with polythene film damage increased numerically with increasing age of bales but did not increase further in older bales aged between 35 and 41 weeks (Table 2.4.5). Of various factors analysed, damage to the polythene film was the only factor that significantly predisposed bales to visible fungal contamination (Table 2.4.6). Bales whose polythene film was damaged (49/100 bales) had a higher ($P<0.001$) proportion of their surface area visibly contaminated with fungi than bales where the film appeared intact. Proportionally, the mean area visibly contaminated with fungi was 0.07 and this varied considerably from bale to bale (range, <0.01 to 0.6). While there was a numerical increase in the proportion of bale surface area contaminated over the months of bale feed-out from November to March and with age of bales (Table 2.4.6), this was not significant ($P=0.272$ and 0.224, respectively). Fungal contamination of bales, as the proportion of surface area affected, tended to decrease with increasing grass wilt time ($P=0.365$) and bales stored in farmyards had approximately 2-fold the proportion of visible fungal contamination on their surface as bales stored in remote fields, but the differences were not significant (Table 2.4.6).

Chemical analyses of baled silage: Fungal coverage on bales showed no correlation with any of the 8 quantitative silage composition variables recorded (Table 2.4.7). Forage DM content (range, 157 - 665 g kg^{-1}) was positively correlated to increasing pH (range, 3.5 - 6.0) and negatively to volatile fatty acids (VFA) (range, 2 - 96 g kg^{-1} DM), lactic acid (range, 2 - 123 g/kg DM) and ammonia-N (range, 2 - 182 g/kgDM) (Table 2.4.7). Other silage variables were variously intercorrelated.

Mycobiota of baled silage: A total of 583 mould and yeast colonies were sampled on 90 bales, resulting in 865 fungal isolates (Table 2.4.8). Single-species fungal cultures were isolated from 377 samples taken from fungal colonies on bales. In the case of other samples taken from fungal colonies, two, three or even four different fungi grew from a single sample. On occasion, two fungi were observed co-existing with each other on the same part of a bale. In most cases the target fungus/fungi sampled from a bale could be recognised in culture, isolated and identified. From 1 to 14 fungal species/genera were isolated from each bale (mean = 4, median = 5 species/bale).

Proportionally, 0.81 of all isolates were identified as mould fungi and the remainder were yeasts (0.19). Six of the most frequently isolated fungi were identified to species level and four more to genus. The most frequently isolated fungus was *Penicillium roqueforti* Thom (0.43) (Table 2.4.8), a mould present on 78 of the 100 bales examined. Other frequently isolated moulds included *Schizophyllum commune,* mucoraceous moulds and *Penicillium paneum* Frisvad. *Fusarium* isolates were identified as *Fusarium culmorum* and *Fusarium avenaceum* from the partial sequence analysis of their rDNA ITS region. Yeasts frequently occurring included *Pichiafermentans, Geotrichum spp.* and *Pichia anomala* (Table 2.4.8). More than 50 other miscellaneous fungal taxa were isolated sporadically in very low numbers from bales and were not identified.

Relative frequencies of isolation of the dominant fungal species on baled silage from November to March are illustrated in Figure 2.4.1A. Numerically, the incidences of *P. roqueforti, S. commune,* and *P. paneum* increased throughout the winter months whereas the incidence of mucoraceous moulds and *Pichia anomala* decreased. *Pichia fermentans* numerically increased in colony numbers from November to February but declined in March. *Geotrichum* did not show any well defined pattern of occurrence. Figure 2.4.1B shows the relative frequencies of occurrence of fungal species on bales following different periods of storage. Numerically, the incidences of *P. roqueforti, P. paneum, Pichiafermentans* and *Geotrichum* increased with bale age (up to weeks 28 to 34), and with the exception of *P. fermentans,* began to decrease in weeks 35 to 41. Mucoraceous moulds numerically decreased with age of bales, whereas *Pichia anomala* decreased gradually from weeks 14 to 34 but showed a higher occurrence in weeks 35 to 41. *S. commune* did not show any well defined pattern of occurrence with bale age; this species was detected in higher numbers in weeks 21 to 27 and 35 to 41 and in lower numbers in weeks 14 to 20 and 28 to 34.

Figure 2.4.2 illustrates the proportions of bale surface area colonised by *P. roqueforti, S. commune* and all other fungi. *P. roqueforti* contaminated an increasing proportion ($P<0.05$) of bale surface area from November to March and occupied a proportionally greater ($P<0.05$) surface area than *S. commune* on bales fed to animals by the end of the silage feeding season in March (Figure 2.4.2A).

Surface areas colonised by *S. commune* were greatest in January and February. The relative areas colonised by *P. roqueforti* and *S. commune* varied with bale age (Figure 2.4.2B), with a higher coverage ($P<0.01$) of *P. roqueforti* compared to *S. commune* in bales aged 28 – 34 weeks old. The ratio of *P. roqueforti:S. commune* surface area on bales was 0.9:1, 0.4:1 , 3.6:1 and 1:1 in the weekly intervals of 14 to 20, 21 to 27, 28 to 34 and 35 to 41, respectively. The two fungi were co-dominant in terms of bale surface occupancy in the oldest bales (35 - 41 weeks). Fungi other than *P. roqueforti* and *S. commune* were less prevalent on older bales and also on bales used in March in comparison to some earlier months.

The occurrence of fungi in relation to harvesting, baling, wrapping and storage practices was analysed using RDA and the resulting bi-plot is presented in Figure 2.4.3. Detrended correspondence analysis indicated that linear forms of ordination were appropriate for the dataset (length of the first gradient = 2.87 standard deviation units of species turnover) therefore RDA was chosen as the most suitable method to analyse the data. A Monte Carlo permutation test (reduced model, 199 permutations) showed that differentiation of the species according to axis 1, and subsequently all canonical axes, was statistically significant ($P<0.05$ and $P<0.01$, respectively), indicating that the variation in the species data-set was not random in relation to variation in the baled silage characteristics shown in Figure 2.4.3. Eigenvalues for the first two axes were 0.26 1 and 0.063 and both axes explained proportionally 0.32 of the variance in the species data. Forward selection of silage characteristics showed that only butyric acid concentration and bale age contributed significantly ($P<0.05$) to the variance found in the species data. These two silage variables explained proportionally 0.19 of the explained variance. Although the remaining silage characteristics were not significant after forward selection, strong trends were evident from the species-environmental bi-plot (Figure 2.4.3). Propionic and butyric acid, ethanol, bale tying, month of bale feed-out, bale age and DM were correlated with axis 1 while bale storage location, lactic and acetic acids, ryegrass and polythene film damage were correlated to axis 2. Ground surface storage type was also correlated with axis 2, but is not considered too important because it is inversely related to bale storage location. Increasing forage DM content was negatively correlated to acetic and butyric acids, and ammonia-N concentrations and increasing lactic acid concentration was inversely correlated to polythene film damage and storage location. *S. commune* was the most common fungus in bales made from ryegrass dominant swards, where visible damage to the polythene film was evident, in bales stored in farmyards and in silage with lower lactic acid concentration. *S. commune* occurrence showed either a weak inverse relationship with the VFA or no relationship at all. All other fungi showed no correlation in regards to polythene film damage, the location where bales were stored or to lactic acid concentration. Occurrence of *P. roqueforti,* and to a lesser extent *Geotrichum,* was positively correlated to increasing propionic and butyric acid concentrations, to month of bale feed-out and to bale age but inversely correlated to ethanol concentration. Low forage DM content favoured the occurrence of *Pichia fermentans, Geotrichum, P. roqueforti* and *P. paneum,* whereas a higher forage DM favoured mucoraceous moulds, *Pichia anomala* and to a lesser extent, *S. commune. P. paneum* was positively correlated to acetic and butyric acids and ammonia-N concentrations. Mucoraceous moulds and *Pichia anomala* were positively correlated to ethanol concentration and with bales tied with netting and inversely correlated to propionic and butyric acid concentrations. *Pichiafermentans* and *Pichiapaneum, Geotrichum* and *Penicillium roqueforti,* and mucoraceous moulds and *P. anomala* were all positively correlated to each other.

Conclusions: Growth of spoilage fungi in baled silage is not of random occurrence but is facilitated where in-bale environments allow the fungi to survive, colonise and reproduce, depending on the nutritional and physical requirements and tolerances of particular species. In bales with four layers of polythene, the periphery of a bale immediately beneath the film is generally not sufficiently anaerobic to prevent fungal growth, as evidenced by the high incidence of visible fungal contamination recorded on the surface of bales in this study. As air ingress *via* damaged film surrounding bales is a major factor permitting fungal growth, it is advisable that farmers ensure more secure anaerobic conditions by, for example, increasing the number of layers of polythene film applied and preventing film damage. Current practices of bale making, transportation and storage need to be modified on many farms in order to prevent damage to the polythene film surrounding bales and thereby reduce the incidence of fungal spoilage and accompanying risk to livestock health.

Table 2.4.1 Baling practices and characteristics of grass crops and bales on farms (*n*=50) surveyed in the Irish midlands from November 2003 to March 2004 (questionnaire responses).

Characteristics	Farmers' answers	Proportion of farms visited	Characteristics	Farmers' answers	Proportion of farms visited
Age of pasture (years)	<10	0.34	Wilt duration (days)[2]	1	0.48
	~10	0.56		2	0.36
	Not reseeded[1]	0.10		~ 3	0.16
Ryegrass dominant	Yes	0.48	Additive applied	No	1.00
	No	0.52			
			Bale tying	Netting	0.60
Grass growth stage at harvest	Stemmy	0.56		Twine	0.40
	Leafy	0.40			
	Both	0.04	Location of wrapping	Site of baling	0.60
				Bale storage area	0.40
Harvest date	May	0.07	No. of film layers [3,4]	4	0.80
	June	0.41			
	July	0.31	Film colour	Black	1.00
	August	0.18			
	September	0.03	Labour source	Baling - contractor	0.76
				Baling - farmer	0.24
Weather at harvest	Dry	0.84		Wrapping - contractor	0.82
	Wet	0.16		Wrapping - farmer	0.18

[1] In living memory; [2]No bales were wilted for less than 1 day; [3]Polythene film; [4]This was the farmers impression and was not verified - proportionally 0.2 farmers failed to answer this question.

Table 2.4.2 Authors' observations on bale storage and on the extent, location and type of damage to bales examined on farms surveyed in the Irish midlands from November 2003 to March 2004

Bale storage		Proportion of farms (*n*=50)	Extent, location and cause of damage on bales		Proportion of bales (*n*=100)
Bale orientation	Flat end[1]	0.66	Polythene damage	Not visibly damaged	0.51
	Curved side[1]	0.30		Visibly damaged[3]	0.49
	Both	0.04			
			Plastic repair patches		0.10
Height of bale storage [2]	Ground tier only	0.88			
	Two tiers	0.08	Location of damage	Shoulder	0.49
	Three tiers	0.04		Barrel	0.47
				Upper end	0.29
Ground surface type	Grass	0.43		Lower end	0.04
	Gravel	0.33			
	Concrete	0.24			
Storage location	Farmyard	0.88			
	Remote field	0.12	Cause of damage[6]	Bird	0.15
				Machinery	0.06
Bale protection[4]	Livestock-proof fencing	0.98		Dog	0.02
	Anti-bird paint	0.12		Cat	0.01
	Anti-bird netting	0		Rodent	<0.01
	Other protection methods[5]	0.04		Other[7]	0.18
	None obvious	0.02			
Bale age (weeks)	14 – 20	0.18			
	21 – 27	0.31			
	28– 34	0.28			
	35-41	0.23			

[1]Touching the ground; [2]Bales examined in detail were sourced only from the ground tier; [3] Including repaired damage with adhesive plastic patches; [4] More than one method was used on some farms; [5]Tyres; wire/twine suspended above bales; wooden pallets placed along-side bales; rat poison laid; [6]Some baales were damaged by several agents; [7]Includes damage due to livestock and other miscellaneous agents.

Table 2.4.3 Chemical composition of bales of grass silage (*n*=100) sampled on 50 farms in the Irish midlands

	Mean	S.D.	Range
Dry matter (DM) (g/kg)	349	114.1	157 - 665
in vitro DM digestibility[1] (g/kg)	644	52.8	469 - 746
Crude protein (g/kgDM)	115	21.1	81 - 175
Ammonia-nitrogen (N) (g/kgN)	58	40.1	2 - 182
pH	4.5	0.38	3.8– 6.0
Ethanol (g/kgDM)	16	9.1	3 -44
Lactic acid (g/kgDM)	33	21.2	2 - 123
Acetic acid (g/kgDM)	12	9.2	<1-42
Propionic acid ($g\ kg^{-1}$ DM)	2	2.0	<1 - 10
Butyric acid (g/kgDM)	11	11.5	<1-48
Total fermentation products (g/kgDM)	74	32.9	11 - 179
Lactic acid/fermentation products (g/g)	0.43	0.156	0.11 -0.73

[1] Not corrected for volatiles; [2]Fermentation products: Lactic, acetic, propionic and butyric acids and ethanol.

Table 2.4.4 Number and sizes of visible fungal colonies on baled grass silage examined on 50 farms in the Irish midlands from November 2003 to March 2004.

Month of bale feed-out and age of bale (weeks)	Number of bales contaminated (number of bales examined)	Number of fungal Colonies (mean±S.D.)[1]	Total surface area of fungal growth (m^2) (mean±S.D.) [1]
November	18 (20)	4±2.7 [a]	0.24±0.238
December	18 (20)	7±3.9	0.50±0.899
January	15 (20)	6±3.8	0. 65±0.495
February	19 (20)	6±4.6	0.50±0.562
March	20 (20)	8±6.6[b]	0. 54±0. 5 80
Total	90 (100)	S.E.D. =1.60 [2]	S.E.D.=0.147[2]
14 – 20	16 (18)	5±2.9	0.23±0.215
21 – 27	26 (31)	5±4.0	0.56±0.813
28 - 34	25 (28)	8±5.7	0.58±0.5 19
35-41	23 (23)	6±5.0	0.46±0.552
Total	90 (100)	S.E.D.=1.39[2]	S.E.D.=0.146[2]

Means with different letters are significantly different by independent sample t-test analysis ($P<0.05$) using square-root transformed data; [1]Calculated for bales showing fungal contamination; [2]S.E.D., standard error of the difference - calculated between bales fed in November and March and between the youngest (14 - 20 weeks) and oldest bales (35 - 41 weeks).

Table 2.4.5 Visible damage to the polythene film on bales of grass silage with increasing bale age

Bale age (weeks)	Number of bales examined	Number of bales damaged	Proportion of bales damaged
14 - 20	18	6	0.33
21 - 27	31	12	0.39
28 - 34	28	17	0.61
35-41	23	14	0.61
Total	100	49	0.49 (mean)

Table 2.4.6 Impact of grass crop, baling, wrapping and storage practices on the proportion of bale surface areas visibly contaminated by fungi

Bale variables[1,2]	Fungal contamination as a proportion of bale surface area (n = no. of bales in category	Bale variables[1,2]	Fungal contamination as a proportion of bale surface area (n = no. of bales in category
Age of pasture (years)[ns]		Labour for wrapping[ns]	
<10	0.048 (n=34)	Contractor	0.070 (n=82)
~10	0.079 (n=56)	Farmer	0.054 (n=18)
Not reseeded[3]	0.065 (n=10)		
Ryegrass dominant[n s]		Bale orientation[ns]	
Yes	0.07 1 (*n*=48)	Flat end[4]	0.069 (*n*=70)
No	0.064 (*n*=52)	Curved side[4]	0.063 (*n*=30)
Harvest date[ns]			
May	0.118 (n=7)	Ground surface storage type[ns]	
June	0.073 (*n*=41)	Grass	0.070 (*n*=43)
July	0.056 (*n*=31)	Gravel	0.058 (*n*=33)
August	0.052 (*n*=18)	Concrete	0.074 (*n*=24)
September	0.084 (*n*=3)		
Weather at harvest [n s]		Storage location [n s]	
Dry	0.069 (*n*=84)	Farmyard	0.072 (*n*=88)
Wet	0.058 (*n*=16)	Remote field	0.035 (*n*=12)
		Polythene film damage[***]	
Wilt duration (days)[n s]			
1	0.083 (*n*=48)	Not visibly damaged	0.036 (*n*=51)
2	0.056 (*n*=36)	Visibly damaged	0.100 (*n*=49)
~3	0.045 (n=16)		
Bale tying[ns]		Month of bale feed-out [n s]	
Netting	0.058 (*n*=60)	November	0.032 (*n*=20)
Twine	0.081 (n=40)	December	0.067 (*n*=20)
		January	0.07 8 (*n*=20)
Location of wrapping[ns]		February	0.077 (n=20)
Site of baling	0.065 (*n*=60)	March	0.083 (n=20)
Bale storage area	0.070 (*n*=40)		
Labour for baling[ns]		Age of bales (weeks)[ns]	
Contractor	0.063 (n=76)	14-20	0.032 (n=18)
Farmer	0.082 (*n*=24)	21-27	0.072 (n=31)
		28-34	0.081 (n=28)
		35-41	0.072 (n=23)

[1] Mean values for each of the bale variable were analysed by the analysis of variance (ANOVA) procedure using square-root transformed data; ns, not significant (*P*>0.05); *** *P*<0.001; [2] To calculate the standard error of the difference (S.E.D.) between any two means the following formula can be used: S.E.D.=¥(E.M.S.(n1 +n2)/(n1 .n2)) where n1 equals the number of observations in one 'category', n2 equals the number of observations in the other 'category' and E.M.S. is the error mean square which equalled 0.008 for all bale variables, except 'polythene film damage' which equalled 0.007; [3] In living memory; [4] Touching

Table 2.4.7. Correlation coefficient *(r)* values of baled silage chemical composition and the proportion of bale surface area visibly contaminated by fungi.

	Fungal cover	DM (g/kg)	pH	Lactic acid	Acetic acid[1]	Propionic acid [1]	Butyric acid [1]	Ethanol
DM (g/kg)	-0.148							
pH	-0.122	0.214*						
Lactic acid (g/kgDM)	0.03 1	-0.565***	-0.551***					
Acetic acid (g/kgDM)[1]	0.070	-0.811***	-0.186	0.542***				
Propionic acid[1] (g/kgDM)	0.053	-0.604***	0.198*	0.047	0.695***			
Butyric acid[1] (g/kgDM)	0.054	-0.595***	-0.007	0.016	0.502***	0.639***		
Ethanol (g/kgDM)	-0.045	-0.165	-0.097	0.131	0.055	-0.012	-0.216*	
Ammonia-N (g/kgN)	-0.051	-0.622***	0.034	0.273**	0.593***	0.662***	0.701***	-0.218*

* *P*<0.05; ** *P*<0.01; *** *P*<0.001; [1]Volatile fatty acids (VFA; g/kgDM)

Table 2.4.8 Fungi isolated from visibly contaminated baled grass silage on Irish midland farms between November 2003 and March 2004.

Fungal genera/species	Number of bales from which isolated	Number of isolates	Proportion of total isolates
Moulds:			
Penicillium roqueforti	78	368	0.43
Schizophyllum commune	43	98	0.11
Mucoraceous moulds	35	71	0.08
Penicillium paneum	22	37	0.04
Fusarium culmorum	10	14	<0.02
Fusarium avenaceum	5	5	<0.01
Trichoderma	7	10	0.01
Unidentified moulds [1]	38	99	0.11
Yeasts:			
Pichiafermentans	36	68	0.08
Geotrichum	23	35	0.04
Pichia anomala	20	30	0.03
Candida boidinii	5	5	0.01
Other yeasts [2]	4	5	0.01
Unidentified yeasts [1]	17	20	0.02
Total	100	865	-

[1] Refers to miscellaneous moulds and yeasts (more than 50 taxa) that were individually of low occurrence; [2] *Issatchenkia orientalis, Pichia membranifaciens* and *Kyuyveromyces lactis*

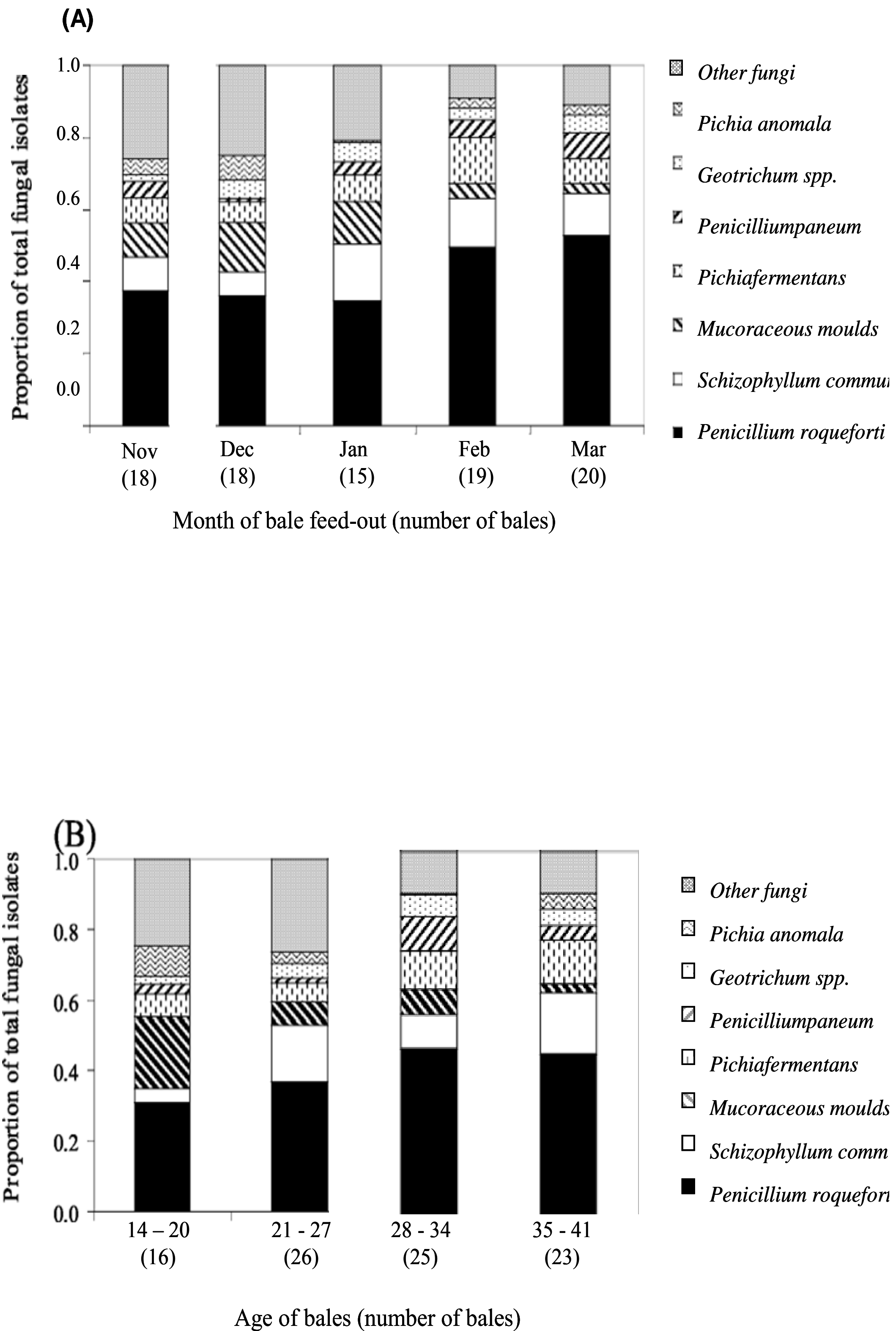


Figure 2.4.1. Relative frequency of isolation of fungal species from colonies on baled grass silage examined on farms in the Irish midlands in relation to (A) month of bale feed-out and (B) age of bales (weeks)

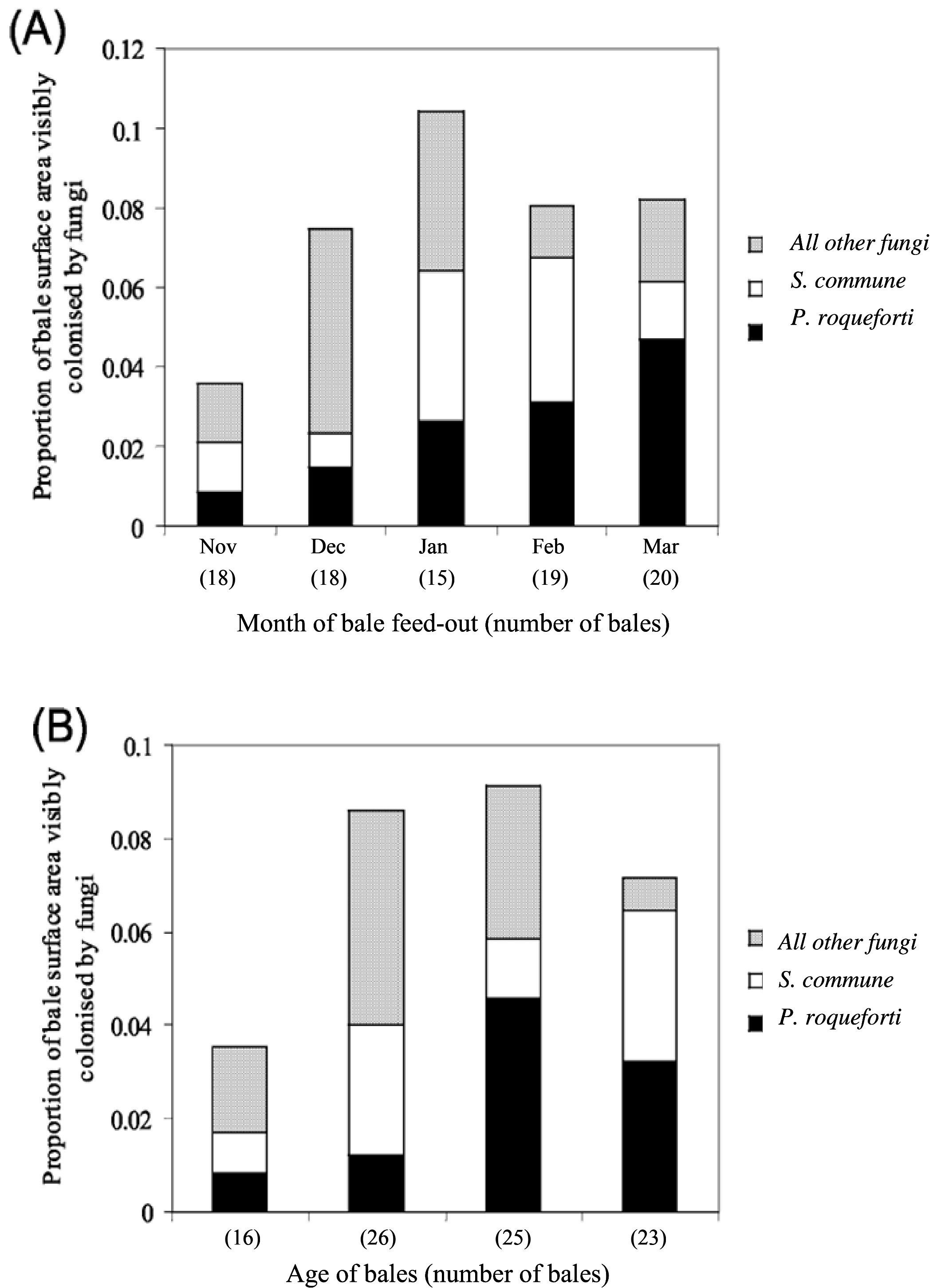


Figure 2.4.2 Colonisation of bale grass silage by *Penicillium roqueforti* and *Schizophyllum commune*. Proportions of bale surface areas colonised in relation to (A) month of bale feed-out and (B) age of bales (weeks). Surface areas colonised by both fungi are underestimated since only pure colonies of each fungus were included in the data set. Mean values of the proportion of bale surface colonised by *P. roqueforti* in relation to month of bale feed-out were analysed by analysis of variance (ANOVA) procedure and differences among the mean proportion of bale surface colonised by *P. roqueforti* and *S. commune* were determined with the independent sample t-test for each month.

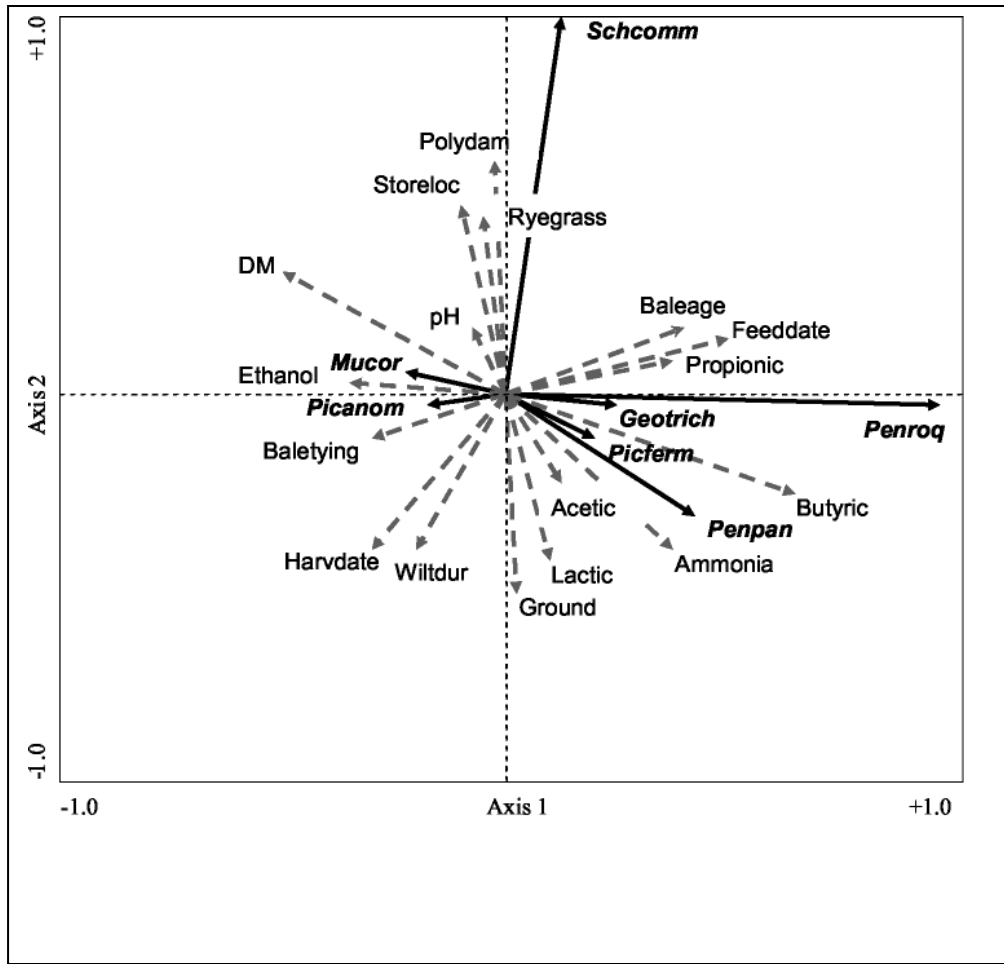


Figure 2.4.3 Redundancy analysis of silage fungi isolated from 90 bales of grass silage from the Irish Midlands. Silage characteristics: ryegrass dominant (Ryegrass), harvest date (Harvdate), wilt duration (Wiltdur), bale tying (Baletying), ground storage surface type (Ground), storage location (Storloc), polythene film damage (Polydam), month of bale feed-out (Feeddate), Age of bales (Baleage), dry matter content (DM), pH (pH), ammonia-N (Ammonia), ethanol concentration (Ethanol), lactic acid concentration (Lactic), acetic acid concentration (Acetic), propionic acid concentration (Propionic) and butyric acid concentration, (Butyric). Fungi: *Penicillium roqueforti* ***(Penroq),*** *Penicillium paneum* ***(Penpan),*** *Pichia fermentans* ***(Picferm),*** *Pichia anomala* ***(Picanom),*** *Schizophyllum commune* ***(Schcomm),*** *Geotrichum* spp. ***(Geotrich)*** and mucoraceous moulds ***(Mucor).*** Data from bales that were not contaminated by fungal growth were not analysed and silage characteristics found to have very short arrows were removed from the biplot. Arrows pointing in the same direction indicate that the corresponding variables are correlated with each other. Long arrows are more important than the short ones. Arrows pointing in the opposite directions are negatively correlated and arrows with an angle of 90 degrees indicate that the two variables are uncorrelated.

Experiment 2.5: Fungal contamination of big-bale grass silage on Irish farms: predominant mould and yeast species and features of bales and silage

[M. O'Brien, O'Kiely, P., Forristal, P.D., Fuller, H.T.]

Experiments 2.3 & 2.4 reported on what fungal species were present on the surface of baled grass silage in the Irish midlands and the extent of their establishment successively during the winter storage/feed-out period. For those studies, all visible fungal colonies on bales were sampled, species isolated and identified. The present study aimed to determine the predominant fungal species present on the surface of baled grass silage in Ireland and to establish the extent of fungal contamination on bales. In addition, the presence of *P. roqueforti* on bales was recorded by sampling at least one *Penicillium*-like colony on each bale based solely on colony appearance. A further objective was to establish how colonisation by the most common fungi related to silage characteristics and on-farm bale management practices.

Materials and methods: *Sample collection:* In February 2004, baled silage was surveyed on 30 farms in each of six regions throughout Ireland (i.e. north west (NW), north midlands (NM), west (W), midlands (M), south west (SW) and south east (SE)), encompassing different farm enterprises and representative of a range of agricultural practices. The location of start and finishing points on routes traversed in each region (latitude, longitude) was: 55°16'N, 07°15'W to 54°28'N, 08°16'W (north west (NW)), 54°07'N, 09°09'W to 54°15'N, 06°57'W (north midlands (NM)), 53°38'N, 08°11'W to 52°38'N, 09°29'W (west (W)), 53°59'N, 07°21'W to 52°40'N, 07°49'W (midlands (M)), 52°30'N, 08°50'W to 52°04'N, 09°30'W (south west (SW)) and 52°30'N, 06°34'W to 51°54'N, 08°29'W (south east (SE)).

Prior to the survey, participants met and together visited a number of farms to ensure standardisation of methods and correct identification of *Penicillium* colonies on bales. The geographical location of bale collections was recorded using the Magellan GPS (Global Positioning System) Pioneer™ satellite navigator (San Dimas, CA, USA). A detailed questionnaire was completed on each farm visited, with information being sought from the farmer on numerous aspects of the baled silage-making process (Table 2.5.1). Additional observations on bale storage practices were recorded on site by the authors (Table 2.5.2). Two bales in readiness for feeding were examined in detail on each farm (n=60 bales/region; total=360 bales). All bales were polythene wrapped and were cylindrical in shape with a nominal diameter and length of approximately 1.2 m. The examination entailed visually scanning the polythene film on each bale for damage and recording the extent, location and cause of damage (if identifiable). On removal of the polythene film, the visible surface area of each fungal colony was quantified as for Experiment 3, and the total area affected by fungal growth was expressed as a proportion of the visible bale surface area. The predominant visible fungal colony, based on the largest area of the bale surface colonised, was sampled as for Experiment 3 and subsequently photographed. On occasions when the predominant fungus was not readily recognised as a *Penicillium* sp., the largest *Penicillium* -like colony was also sampled (i.e. an additional second sample was taken per bale).

Fungal samples were collected either by touching a sporulating colony with a sterile moist cotton swab or by placing a small fragment of foliage colonised with fungal material into an individual sterile container that was stored at 4 °C. Additionally, bale collections on the farms were also assessed for the occurrence of the macrofungus *Schizophyllum commune.* The proportion of bales on each farm where *S. commune* was visibly evident protruding through the polythene film was recorded.

Results: In summer 2003, a mean of 272 bales/farm were harvested on the 180 farms included in the survey and, on average, 92 bales/farm remained to be fed when farms were visited in February 2004. The harvested herbage was from permanent temperate grassland of mixed botanical composition. Bales were stored from 25 to 44 weeks (mean and median = 36 and 37 weeks, respectively) before being fed to livestock. Results of a questionnaire regarding production of baled silage are collated in Table 2.5.1 and the authors' observations on bale storage and polythene film damage are presented in Tables 2.5.2 and 2.5.3. Both sets of survey results together with silage chemical analyses (Table 2.5.4) were used in CCA.

Visible fungal contamination of bales: Fungal growth was visibly present on 331 of the 360 bales examined (Table 2.5.5). The proportion of bales affected ranged from 0.78 (47/60) in the north west (NW) to 0.98 (59/60) in the south west (SW) of Ireland. On average, there were five visible fungal colonies on each affected bale and this ranged from 1 to 21 colonies per bale. The number of visible fungal colonies on bales was higher (P<0.05) in the west (W) and midlands (M) than in the NW, north midlands (NM) and the south east (SE). There

was also a higher number (P<0.05) of fungal colonies on bales in the SW compared to the SE (Table 6.5). Surface areas colonised by fungi were less on bales fed to livestock in the NM and SE compared to the NW, W and M (P<0.05) and also less on bales examined in the SE compared to the SW (P<0.05). Bales of all age groups did not differ in the number of visible fungal colonies present nor in the extent of fungal colonisation (P>0.05) (Table 2.5.5). Bale age was calculated from the time bales were wrapped to when they were examined by the surveyors.

Fungal spoilage and features of harvesting, baling, wrapping and storage: The proportion of bales with polythene film damage was numerically similar regardless of the age of bales (Table 2.5.6). Expressed as a proportion of the visible surface area, the mean and median area visibly contaminated with fungi was 0.060 and 0.033, respectively and this varied considerably from bale to bale (range, <0.01 to 0.82). Of various factors analysed, weather at harvest, region location and damage to the polythene film were the most significant factors that were associated with visible fungal contamination (Table 2.5.6). Bales harvested in dry weather had less (P<0.01) visible fungal contamination than bales harvested in wet conditions. Bale susceptibility to visible fungal contamination differed in the different regions surveyed (P<0.001). For example, the proportion of the bale surface area contaminated with fungal growth was lower on bales in the SE compared to the NW, W, M and SW and lower in the NM than the W and M (P<0.05). Bales whose polythene film was damaged (179/3 60 bales) had a higher (P<0.001) proportion of their surface area visibly contaminated with fungi than bales where the film appeared intact. This damage was caused primarily by birds, machinery and cats and affected the barrel and upper shoulder of bales to the greatest extent (Table 2.5.3). Although a lower number of bales (72/170) stored on their flat end were observed to have visible polythene damage compared to bales stored on their curved side (107/190), bales on their flat end had a higher proportion of their surface area contaminated by fungi (P<0.05) (Table 2.5.7). Wilt duration was also a significant factor that was associated with fungal contamination on bales (P<0.05), but there was no obvious trend evident as the multiple comparison test failed to detect differences between any pair of means. Other management practices that were associated (P<0.05) with a reduction in visible fungal contamination included the use of netting rather than twine, transporting and wrapping bales close to the bale storage area, wrapping and baling by the farmer rather than employing a contractor, and stacking bales no higher than two tiers high.

Fungal contamination of bales stored on their flat end was not contingent on the ground surface type, but the type of surface was important for bales stored on their curved side, with half the amount (P<0.05) of fungal contamination for bales stored on concrete compared to either grass or gravel (Table 2.5.8). Height of bale storage (i.e. number of tiers) had no influence of the proportion of the bale surface contaminated for bales stored on their curved side (P>0.05) (Table 2.5.6). *Chemical analyses of baled silage and relationship with fungal contamination:* Of the eight quantitative silage composition variables recorded in this study (Table 2.5.9), fungal coverage on bales showed a significant correlation only with butyric acid concentration (P<0.01). The mean forage DM content in bales in the NW was 251g/kg and this was lower (P<0.001) than in bales from all other regions surveyed (mean range, 307 – 356 g/kg). Forage DM content (range, 126 - 650 g/kg) was positively correlated to increasing pH (range, 3.8 - 7.6) and negatively to VFA (range, 2 - 141 g/kg DM), lactic acid (range, 1 - 123 g/kg DM) and ammonia-N (range, 1 - 410 g/kgN) (Table 2.5.9). Other silage variables were variously intercorrelated.

Mycobiota of baled silage: With regards to the predominant fungus on bales, a total of 331 mould and yeast visible colonies were sampled on 331 bales, resulting in 345 fungal isolates (Table 2.5.10). Single-species fungal cultures were isolated from 201 samples taken from the largest fungal colonies on bales. In the case of other samples taken from fungal colonies, two, three or even four different fungi grew from a single sample. On occasion, two fungi were observed co-existing with each other on the same part of a bale. In most cases the target fungus/fungi sampled from a bale could be recognised in culture, isolated and identified. However, the identity of the predominant fungal colony on 34/331 bales could not be assigned from the mixed mycobiota isolated in each case.

In the majority of occasions when the predominant fungus was identified, proportionally, 0.81 of these fungi were identified as mould and the remainder (0.19) were yeasts. Seven of the predominant fungi were identified to species level and two more to genus. The predominant fungus on bales was *Penicillium roqueforti* Thom (0.42) (Table 2.5.10) i.e. it formed the largest colonies on 146 of the 360 bales examined. This fungus was present on a total of 175 of the 360 bales examined based on the sampling of at least one colony recognised as *Penicillium* on each individual bale. Other frequently isolated fungi included

Schizophyllum commune and *Penicillium paneum* Frisvad; *Fusarium* isolates were identified as *Fusarium culmorum* from the partial sequence analysis of their rDNA ITS region. Yeasts frequently occurring included *Pichia fermentans, Geotrichum spp.* and *Pichia anomala* (Table 2.5.10). Additionally, *S. commune* was visibly protruding through the polythene film on 106 of the 180 bale collections on farms visited. More than 50 other miscellaneous fungal taxa were isolated sporadically in very low numbers from bales and were not identified.

Fungal colony size and appearance on bales was very variable (Table 2.5.10). *P. roqueforti* and *P. paneum* colonies were indistinguishable from each other on bales as both were characteristically blue to green-coloured, and had a diffuse to floccose felt appearance. Occasionally, a whitish-coloured diffuse mycelial perimeter could be observed on the colonies. Smaller colonies were more often observed entirely with whitish-coloured diffuse mycelia whereas, due to sporulation, larger colonies were blue to green in colour. *S. commune* varied in appearance from white to cream-coloured floccose felt to patches of a dense compact mat, white to beige to brown in colour with nodules of differentiating tissue. The mean colony size of *S. commune* was larger (*P*<0.001) than *P. roqueforti* colonies on bales (Table 2.5.10). *F. culmorum* colonies appeared pink to whitish with a diffuse to floccose felt texture and *Trichoderma* colonies were blue to green-coloured, diffuse, with a rather rough granular texture. Yeast colonies *(P. fermentans* and *P. anomala)* were white to cream-coloured diffuse powdery appearance to enveloping the bale in a thin surface film. *Geotrichum* appeared mould-like on bales and colonies varied in colour from white to grey to cream and appeared diffuse, floccose or in a compact mat and occasionally formed a surface film.

Visible fungal-contaminated silage had a higher pH (*P*<0.001) (mean = 6.7, median = 7.0) than silage from the same bales that were free of visible fungal contamination (mean and median = pH 4.5). However, the pH of mould-contaminated silage (mean, 6.9, median 7.1; n = 70 bales) was, on average, numerically greater than yeast-contaminated silage (mean, 5.1, median = 4.3; n = 9 bales). The pH of silage contaminated with *P. roqueforti* was higher (*P*<0.05) than silage contaminated by *S. commune.*

Relative frequencies of isolation of the predominant fungal species on baled silage from each region are illustrated in Figure 2.5.1A. Numerically, the incidences of some fungi varied among regions. In the most northern region (NW), *S. commune* was not detected and numbers were also lower in the second most northern region (NM) compared to the more southern regions (W, M, SW and SE); *S. commune* occurrence in these four southern regions were very similar (between 0.29 to 0.30 of all fungal isolates). *P. roqueforti* was detected as the predominant fungal colony in higher numbers on bales in the NM but it occurred less frequently in the NW than in all other regions. In the four southern regions (W, M, SW and SE), *P. roqueforti* occurrence ranged from 0.36 to 0.42 of all fungal isolates. *P. paneum* numbers were relatively similar in all regions and ranged from 0.02 to 0.07 of all fungal isolates. The incidence of *Pichia fermentans* was greatest in the NW but decreased progressively from north to south and was not identified as the predominant fungus on any bales in the SE. *Geotrichum* did not show any well defined pattern of occurrence.

Figure 2.5.1B shows the relative frequencies of occurrence of fungal species on bales following different periods of storage. Numerically, the incidences of *P. roqueforti* and *Geotrichum* increased with bale age (up to weeks 35 to 39) and then plateaued in weeks 40 to 44. *S. commune* and *P. fermentans* did not show any well defined pattern of occurrence with bale age, however *P. fermentans* was detected in higher numbers in weeks 30 to 34 and 40 to 44 and in lower numbers in weeks 25 to 29 and 35 to 39. *P. paneum* was not detected in the very youngest bales and decreased numerically in occurrence from weeks 30 to 34 onwards.

The occurrence of fungi in relation to harvesting, baling, wrapping and storage practices was analysed using CCA and the resulting bi-plot is presented in Figure 2.5.2. DCA indicated that unimodal forms of ordination were appropriate for the dataset (length of the first gradient = 3.131 standard deviation units of species turnover) therefore CCA was chosen as the most suitable method to analyse the data. A Monte Carlo permutation test (reduced model, 199 permutations) showed that differentiation of the species according to axis 1, and subsequently all canonical axes, was statistically significant (*P*<0.0 1), indicating that the variation in the species data-set was not random in relation to variation in the baled silage characteristics shown in Figure 2.5.2. Eigenvalues for the first two axes were 0.357 and 0.149 and both axes explained proportionally 0.17 of the variance in the species data. Forward selection of silage characteristics showed that only region location (*P*<0.01), lactic acid concentration (*P*<0.01), butyric acid concentration (*P*<0.05) and weather at harvest (*P*<0.05) contributed to the found variance in the species data. These four silage variables

explained proportionally 0.63 of the explained variance. Although the remaining silage characteristics were not significant after forward selection, strong trends were evident from the species-environmental bi-plot (Figure 2.5.2). Polythene damage, DM, region location, bale orientation, lactic and acetic acid concentrations and ammonium-N concentration were correlated with axis 1 while ryegrass, bale tying, ethanol concentration, bale storage location, butyric and propionic acid concentrations and weather at harvest were correlated to axis 2. Increasing forage DM content was negatively correlated to bale orientation, acetic and lactic acids, and ammonia-N concentrations and increasing lactic acid concentration was inversely correlated to bale storage height and pH. Region location, plastic damage and DM, storage height and pH, and acetic and lactic acids and ammonium-N concentrations were all correlated with each other. *S. commune* was the most common fungus in bales stored more than one tier high, where visible damage to the polythene film was evident and in silage with a high pH and DM content, lower lactic and acetic acid and ammonia-N concentrations. *S. commune* occurrence showed either a weak inverse relationship with VFA or no relationship at all. All other fungi showed no correlation in regards to polythene film damage, the location where bales were stored or to lactic acid concentration. Occurrence of *P. roqueforti* and *P. paneum* was positively correlated to increasing propionic and butyric acid concentrations and to bales harvested in dry weather. *P. fermentans* occurred on bales harvested from forage of a lower DM content later in the summer, stored on their ends, where polythene film did not become damaged during storage and was more common in the northern half of the country (i.e. NW and NM) than in the south. *P. roqueforti* and *P. paneum* favoured similar conditions, whereas *S. commune* and *P. fermentans* preferred different conditions to each other and to the Penicillia.

Conclusions: This is the first extensive survey of mould and yeast growth on baled grass silage in Ireland. Visible fungal contamination was shown to be widespread on baled silage on the 180 farms surveyed. *P. roqueforti* has an almost ubiquitous distribution in bale collections, whereas *S. commune,* and to a lesser extent *P. fermentans,* is strongly influenced by the DM content of silage. A less extensive wilt may make bales less susceptible to *S. commune,* but will do little to prevent growth of *P. roqueforti* which is of more concern because of its ability to produce potentially harmful mycotoxins in silage. As air ingress *via* damaged film surrounding bales is a major factor permitting fungal growth, it is essential for livestock owners ensure that the polythene film applied to bales does not become damaged, thereby promoting a more extensive LAB fermentation while at the same time limiting the supply of oxygen that would favour the growth of spoilage fungi.

Table 2.5.1. Baling practices and characteristics of grass crops and bales on farms (n = 180) surveyed in Ireland during February 2004 (questionnaire responses).

Characteristics	Farmers' answers	Proportion of farms visited	Characteristics	Farmers' answers
Age of pasture (years)	<10	0.47	Additive applied	No
	>10	0.40		Yes
	Not reseeded [1]	0.14		
			Bale tying	Netting
Ryegrass dominant sward	Yes	0.47		Twine
	No	0.53		
			Location of wrapping	Site of baling
Grass growth stage at harvest	Stemmy	0.68		Bale storage area
	Leafy	0.31		
	Both	0.02		
Harvest date	May	0.08	No. of film layers [2, 3]	4
	June	0.49		
	July	0.29	Film colour[2]	Black
	August	0.10		White
	September	0.04		
			Labour source	Baling - contractor
Weather at harvest	Dry	0.78		Baling - farmer
	Wet	0.22		Wrapping - contractor
				Wrapping - farmer
Wilt duration (days)	<1	0.06		
	1	0.46		
	2	0.34		
	3	0.10		
	>3	0.04		

[1]In living memory; [2]Polythene film; [3]This was the farmers impression and was not verified; proportionally 0.21 farmers failed to answer this question.

Table 2.5.2. Authors' observations on bale storage and on the extent of damage to bales examined on farms surveyed in Ireland during February 2004.

Bale storage		Proportion of farms (n= 180)	Extent of visible damage on bales		Proportion of bales (n=360)
Bale orientation	Flat end[1]	0.45	Bale protection[2]	Livestock-proof fencing	0.94
	Curved side[1]	0.49		Anti-bird netting	0.04
	Both	0.06		Anti-bird paint	0.09
				Tyres	0.02
Height of bale storage	Ground tier only	0.64		Other protection methods [3]	0.04
	Two tiers	0.14		None obvious	0.02
	Three tiers	0.20			
	Four tiers	0.02	Bale age (weeks)[4]	25 - 29	0.11
				30-34	0.30
Ground surface type	Grass	0.34		35-39	0.39
	Gravel	0.44		40- 44	0.20
	Concrete	0.22			
			Film damage	Not visibly damaged	0.51
Storage location	Farmyard	0.81		Visibly damaged [5]	0.49
	Remote field	0.19			

[1]Touching the ground; [2]More than one method was used on some farms; [3]Tyres; wire/twine suspended above bales; wooden pallets placed along-side bales; rat poison laid; [4]Duration from harvesting to survey; [5]Including repaired damage with adhesive plastic patches.

Table 2.5.3. Location of damage caused by different agents to bales of grass silage on farms surveyed in Ireland during February 2004.

Damage location[2]	Cause and extent of damage to the polythene film on bales (n= 179 bales damaged)[1]					
	Bird (n=98 bales)	Machinery (n=37 bales)	Cat (n=13 bales)	Rodent (n=8 bales)	Other[3] (n=54 bales)	Proportion of visible damage points on bales (n=248)
Upper end	++	+	+	+++	+	0.15
Lower end	-	++	-	-	+	0.03
Upper barrel	++++	++++	++	++	++	0.44
Lower barrel	+	+++	+++	+++	+++	0.18
Upper shoulder	++	+	++++	-	++	0.16
Lower shoulder	+	+	-	+++	+	0.04

-:none detected; +: 0– 0.09; ++: 0.1– 0.24; +++: 0.25– 0.39; ++++: 0.4– 0.55 (as a proportion of visible damaged points on bales).

[1]Polythene-film surrounding bales was occasionally damaged in more than one location and by more than one agent;

[2]Is contingent on the orientation of the bale when examined;

[3]Includes damage due to livestock and other miscellaneous agents.

Table 2.5.4. Chemical composition of bales of grass silage (n = 360) sampled on 180 farms in Ireland during February 2004.

	Mean	S.D.	Range
Dry matter (DM; g/kg)	321	109.9	126 - 650
in vitro DM digestibility[1] (g/kg)	644	52.8	469 - 746
Crude protein (g/kgDM)	115	21.1	81 - 175
Ammonia-nitrogen (N) (g/kgN)	58	40.1	2 - 182
pH	4.5	0.38	3.8– 6.0
Ethanol (g/kgDM)	16	9.1	3 -44
Lactic acid (g/kgDM)	33	21.2	2 - 123
Acetic acid (g/kgDM)	12	9.2	<1-42
Propionic acid (g kg^{-1} DM)	2	2.0	<1 - 10
Butyric acid (g/kgDM)	11	11.5	<1-48
Total fermentation	74	32.9	11 - 179
Lactic acid/fermentation products (g/g)	0.43	0.156	0.11 -0.73

[1]Fermentation products: Lactic, acetic propionic and butyric acids, and ethanol

Table 2.5.5. Number and sizes of visible fungal colonies on baled grass silage examined on 180 farms in Ireland during February 2004.

Region location and age of bales (weeks)	Number of bales contaminated (number of bales examined)	Number of fungal colonies (mean±S.D.) [1, 2]	Total surface area of fungal growth (m^2) (mean±S.D.) [1, 2]
North west (NW)	47 (60)	4±2.4 ^{ac}	0.55±0.79 1 ^{bc}
North midlands (NM)	58 (60)	4±2.4 ^{ac}	0.21±0.161 ^{abc}
West (W)	58 (60)	7±4.1 ^{bc}	0.51±0.669 ^{bc}
Midlands (M)	54 (60)	7±5.1 ^{bc}	0.56±0.543 ^{bc}
South west (SW)	59 (60)	6±3.3 ^{c}	0.43±0.453 ^{c}
South east (SE)	55 (60)	3±1.8 ^{a}	0.15±0.231 ^{a}
Total	331 (360)	***	***
25– 29	34 (39)	5±4.5	0.47±0.520
30– 34	99 (108)	5±3.8	0.33±0.359
35 - 39	130 (139)	5±3.5	0.38±0.464
40 -44	68 (74)	5±3.7	0.49±0.822
Total	331 (360)	ns	ns

[1]Calculated for bales showing fungal contamination; [2]Mean values for each bale variable were analysed non-parametrically using the Kruskall-Wallis procedure; ns, not significant ($P > 0.05$); ***$P < 0.001$. Mean values with a common letter superscript within each data set do not differ significantly as determined by the Tukey multiple comparison test.

Table 2.5.6. Visible damage to the polythene film on bales of grass silage with increasing bale age; bales examined on 180 farms in Ireland during February 2004

Bale age (weeks)	Number of bales examined	Number of bales with visible damaged to film	Proportion of bales with visible damaged to film
25 - 29	39	20	0.51
30-34	108	52	0.48
35 - 39	139	71	0.51
40 - 44	74	36	0.49
Total	360	179	0.49 (mean)

Table 2.5.7. Impact of grass crop, baling, wrapping and storage practices on the proportion of bale surface areas visibly contaminated by fungi; observations based on responses to a questionnaire completed by 180 farmers in Ireland in February 2004

Bale variables[1]	Fungal contamination as a proportion of the bale surface area (n=numbers of bales in category)	Bale variables[1]	Fungal contamination as a proportion of the bale surface area (n=numbers of bales in category)
Age of pasture (years)[ns]		Labour for wrapping[*]	*P=0-045*
<10	0.056 (*n*=168)	Contractor	0.062 (*n*=261)
>10	0.064 (n=142)	Farmer	0.052 (*n*+99)
	0.056 (*n*=50)		
		Bale orientation*	*P=0.014*
Ryegrass dominant[ns]		Flat end	0.067 (*n*-170)
Yes	0.060 (*n*=170)	Curved side[3]	0.053 (*n*=190)
No	0.059 (*n*=190)		
		Height of bale storage*	*P=0.033*
Harvest date[ns]		Ground tier only	0.060 (*n*=290)
May	0.104 (*n*=30)	Two tiers	0.038 (*n*=51)[a]
June	0.055 (*n*=174)	≥ Three tiers[4]	0.098 (*n*=19)[b]
July	0.056 (*n*=104)		
August	0.058 (*n*=36)	Storage ground surface type[ns]	
September	0.050 (*n*=16)	Grass	0.059 (*n*=123)
		Gravel	0.065 (*n*=158)
Weather at harvest**	*P=0.005*	Concrete	0.047 (*n*=79)
Dry	0.055 (*n*=281)		
Wet	0.076 (*n*=79)	Storage location[ns]	
		Farmyard	0.058 (*n*=293)
Wilt duration (days)*	*P=0.017*	Remote field	0.067 (*n*=67)
<1	0.033 (*n*=46)		
1	0.071 (*n*=168)	Polythene film damage***	
2	0.052 (*n*=104)	Not visibly damaged	0.044 (*n*=181)
3	0.056 (*n*=28)	Visibly damaged	0.075 (*n*=179)
>3	0.075 (*n*=14)		
		Region***	*P = <0.001*
	P=0.0021	North west (NW)	0.066 (*n*=60)[a]
	0.057 (*n*=255)	North midlands (NM)	0.035 (*n*=60)[ab]
	0.065 (*n*=105)	West (W)	0.084 (*n*=60)[ac]
		Midlands (M)	0.079 (*n*=60)[ac]
Location of wrapping*	*P=0.041*	South west (SW)	0.070 (*n*=60)[a]
Site of baling	0.061 (*n*=216)	South east (SE)	0.023 (*n*=60)[b]
Bale storage area	0.056 (*n*=144)		

[1] Mean values for each bale variable were analysed non-parametrically using either the Mann-Whitney or Kruskall-Wallis procedure; ns, not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Mean values with a common letter superscript within each bale variable do not differ significantly as determined by the Tukey multiple comparison test; [2]In living memory; [3]Touching the ground; [4]Data from the third and fourth tiers were combined.

Table 2.5.8. Impact of bale storage practices on the proportion of bale surface areas visibly contaminated by fungi; bales examined on 180 farms in Ireland during February 2004

Bale variable[1]	Fungal contamination as a proportion of bale surface area (n=number of bales in each category)	
	Curved side (n=190)	Flat end (n=170)
Ground surface storage type		
Grass	0.058 (n=51)	0.059 (n=72)
Gravel	0.060 (n=97)	0.074 (n=60)
Concrete	0.029 (n=42)	0.067 (n=38)
Significance	*	ns
Height of bale storage[2]		
Ground tier only	0.051 (n=122)	n=168
Two tiers	0.039 (n=49)	(n=2)
~ Three tiers	0.098 (n=19)	-
Significance	ns	-

[1] Mean values for each bale variable were analysed non-parametrically using the Kruskall-Wallis procedure; ns, not significant *(P* > 0.05); *P < 0.05; [2] Bales on their flat end were predominantly stored on the ground and therefore data were not amenable to statistical analysis.

Table 2.5.9. Correlation coefficient *(r)* values of baled silage chemical composition and the proportion of bale surface area visibly contaminated by fungi; silage sampled from 360 bales during February 2004.

	Fungal cover	DM (g/kg)	pH	Lactic acid	Acetic acid[1]	Propionic acid [1]	Butyric acid [1]	Ethanol
DM (g/kg)								
pH		**0.24**						
Lactic acid (g/kgDM)		**-0.56**	**-0.69**					
Acetic acid (g/kgDM)[1]		**-0.76**	**-0.27**	**0.54**				
Propionic acid[1] (g/kgDM)		**-0.59**		**0.21**	**0.68**			
Butyric acid[1] (g/kgDM)	0.15	**-0.47**		0.16	**0.47**	**0.65**		
Ethanol (g/kgDM)		-0.14	**-0.28**	**0.29**	0.14			
Ammonia-N (g/kgN)		**0.62**		**0.36**	**0.61**	**0.54**	**0.61**	

P <0.001 shown in old, otherwise *P*< 0.01. [1]Volatile fatty acids (VFA)

Table 2.5.10. Predominant fungi isolated from visibly contaminated baled grass silage in Ireland and the appearance of fungi *in situ;* 360 bales examined on 180 farms in Ireland during February 2004.

Fungal genera/species	[1] No. of bales on which predominant	Proportion of bales	Colony size (m^2) (mean±S.D.)	Colony colour on bale surface								Colony appearance and texture on bale					pH (Number of colonies) [4]
				Bl	Gn	Gy	W	Be	Bn	C	P	Diff	Mat	Floc	Film	Powd	
Moulds:																	
Penicillium roqueforti	146	0.42	0.13±0.135 a	**¥**	**¥**	¥	**¥**			¥		**¥**	¥	**¥**			7.0±1.10 (n=36) [a]
Schizophyllum commune	72	0.21	0.34±0.404 b	**¥**				**¥**	**¥**	**¥**		**¥**	**¥**	**¥**			6.7±0.76 (n=28) [b]
Penicilliumpaneum	16	0.05	0.21±0.381	¥	**¥**	¥	**¥**	¥		¥		**¥**	¥	**¥**			6.7±2.01(n=3)
Fusarium culmorum	4	0.01	0.25±0.206				**¥**			**¥**	**¥**	**¥**	**¥**	**¥**			nd
Trichoderma	3	0.01	0.19±0.196	**¥**	**¥**		**¥**			**¥**		**¥**					7.2 (n=1)
Coprinus	1	<0.01	4.51±0.0	**¥**					**¥**	**¥**		**¥**					7.5 (n=1)
Unidentified moulds [2]	9	0.03	0.09±0.083	**¥**		**¥**	**¥**					**¥**	**¥**	**¥**			6.9 (n=1)
Yeasts:																	
Pichiafermentans	36	0.10	0.19±0.178	**¥**						**¥**		**¥**	¥	¥	**¥**	**¥**	3.8±1.60 (n=5)
Geotri chum	13	0.04	0.21±0.250	¥			**¥**	¥	¥	**¥**		**¥**	**¥**	**¥**	¥		6.9±0.69 (n=2)
Pichia anomala	4	0.01	0.05±0.060	**¥**						**¥**		**¥**				**¥**	4.9±0.90 (n=2)
Candida boidinii	1	<0.01	0.14±0.0				**¥**								**¥**		nd
Unidentified yeasts [2]	6	0.02	0.98±1.76	¥			**¥**			¥		¥	¥		**¥**	**¥**	nd
Mixed mycobiota[3]	34	0.10	0.29±0.562	**¥**	**¥**	**¥**	**¥**	**¥**		**¥**		**¥**	**¥**	**¥**	**¥**	**¥**	6.9±1.04 (n=7)
Total	345																

P. roqueforti and *S. commune* mean colony size and pH of fungal-contaminated silage were analysed non-parametrically using the Mann-Whitney procedure. Mean values with a different letter superscript differ significantly; $P < 0.05$ (pH); $P < 0.001$ (colony size). [1] On occasions, two fungi were observed coexisting with each other on the same part of a bale. [2] Refers to miscellaneous moulds and yeasts (more than 50 taxa) that were individually of low occurrence. [3]>1 fungus was isolated but the predominant fungus/fungi was not obvious. [4] Refers to the pH of the fungal-contaminated silage of each individual species/genera. Abbreviations: Bl, blue; Gy, grey; Gn, green; W, white; Be, beige; Bn, brown; C, cream; P, pink; Diff, diffuse or loose, barely visible mycelium/growth over the bale surface; Mat, compact mat of very tightly packed mycelium/growth; Floc, floccose or cottony-like mycelium; Film, surface film of slimy growth on bale surface; Powd, powdery-like growth on bale surface; tick marks in bold **(¥),** common colony colour, appearance and textures observed frequently; unbolded tick marks (¥), colony colour, appearance and textures observed only occasionally; nd, not determined.

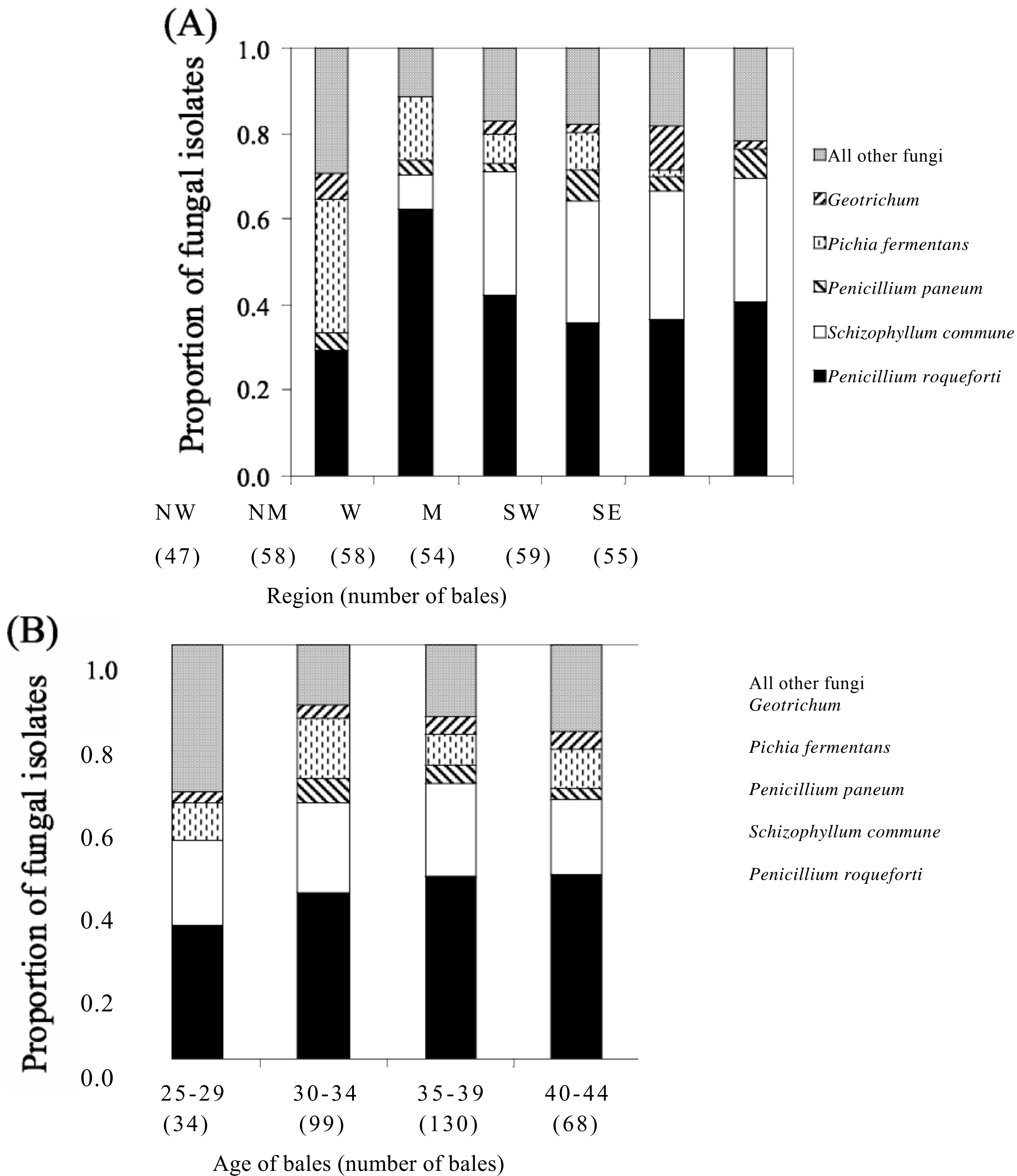


Figure 2.5.1. Relative frequency of isolation of the fungal species from the predominant colonies on baled grass silage examined in the Republic of Ireland in relation to (A) region and (B) age of bales (weeks). All other fungi refer to other moulds and yeasts that were individually of low occurrence such as *Fusarium culmorum, Trichoderma, Coprinus, Pichia anomala, Candida boidinii,* other unidentified yeasts and moulds and mixed mycobiota. Region abbreviations are: NW=north-west, NM=north-midlands, W=west, M=midlands, SW=south-west, SE=south-east.

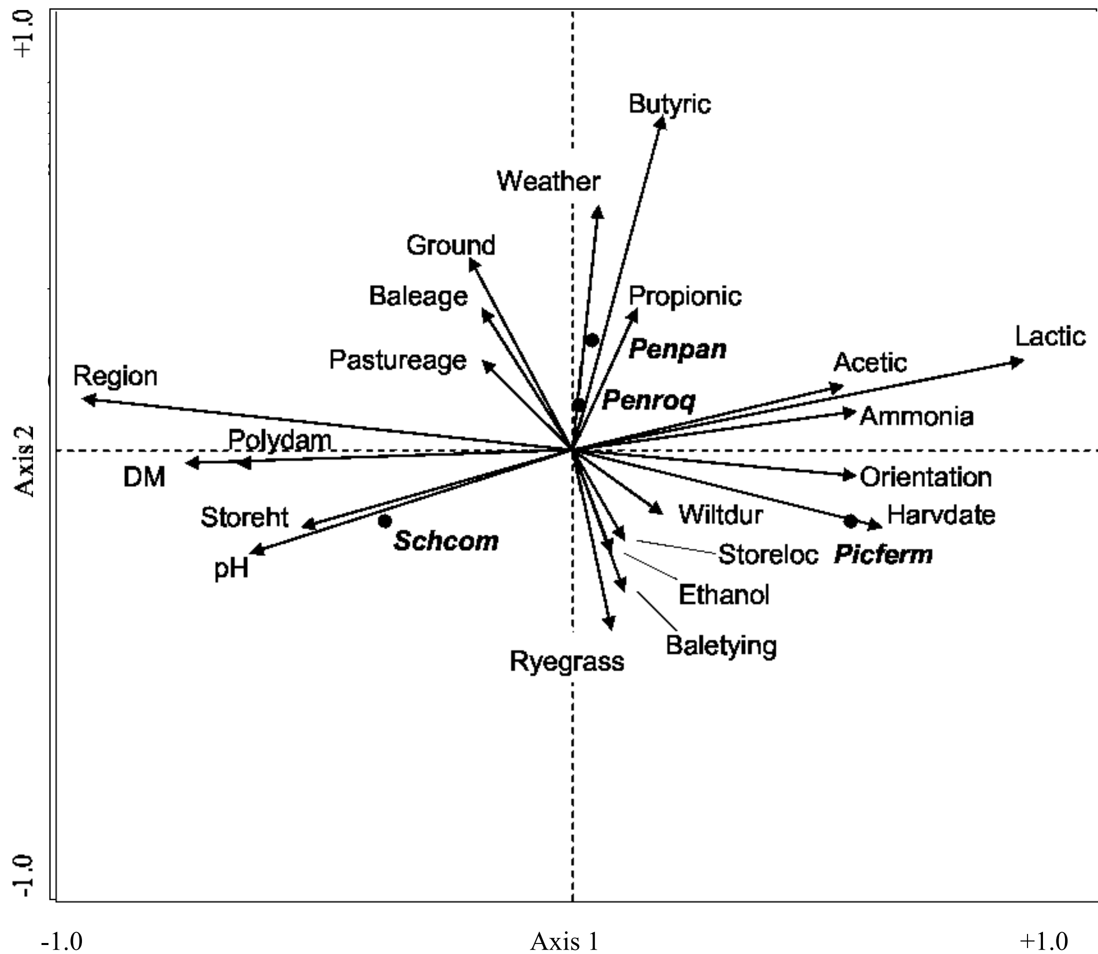


Figure 2.5.2. Canonical correspondence analysis of silage fungi isolated from 260 bales of grass silage sampled in the Republic of Ireland during February 2004. Silage characteristics: age of pasture (pastureage), ryegrass dominant (Ryegrass), harvest date (Harvdate), weather at harvest (weather), wilt duration (Wiltdur), bale tying (Baletying), bale orientation during storage (orientation), height of bale storage (storeht), storage ground surface type (Ground), storage location (Storeloc), polythene film damage (Polydam), region location (Region), Age of bales (Baleage), dry matter content (DM), ammonia-N (Ammonia), pH (pH), ethanol concentration (Ethanol), lactic acid concentration (Lactic), acetic acid concentration (Acetic), propionic acid concentration (Propionic) and butyric acid concentration, (Butyric). Fungi: *Penicillium roqueforti* ***(Penroq),*** *Penicillium paneum* ***(Penpan),*** *Pichia fermentans* ***(Picferm)*** and *Schizophyllum commune* ***(Schcom).*** Data from bales that were not contaminated by fungal growth or where the largest fungal colony was not identified as either of above four fungal species were excluded from the analysis. Silage characteristics found to have very short arrows were removed from the bi-plot.

Experiment 2.6: Mycotoxins and other secondary metabolites produced *in vitro* by *Penicillium paneum* Frisvad and *Penicillium roqueforti* Thom isolated from baled grass silage in Ireland

[M. O'Brien, O'Kiely, P., Forristal, P.D., Fuller, H.T.]

In previous experiments evidence has been presented that fungal contamination of baled grass silage in Ireland is widespread; up to 90 % of bales examined had visible fungal growth present. The common occurrence of moulds such as *Penicillium roqueforti* and to a lesser extent *P. paneum* raises the possibility that these known toxigenic fungi might be producing toxins in silage. This study aimed to characterise the secondary metabolite profiles of *P. roqueforti* and *P. paneum* isolated from baled grass silage in Ireland, to induce *P. paneum* isolates to produce patulin by supplementing the growth medium with trace metals, e.g. manganese, and to assess the ability of these *P. paneum* isolates to produce patulin after storage for 30 weeks. Another objective was to screen visually mouldy and visually non-mouldy grass silage samples for secondary fungal metabolites.

Materials and methods: *Sample collection and isolate selection:* In previous experiments, a total of 2277 visible fungal colonies were enumerated on baled silage and 1190 fungal colonies were sampled and later identified. Of this total, 830 were identified as *P. roqueforti* and 78 as *P. paneum* by their macro- and micromorphology features, using appropriate identification keys. Approximately 10 % of the *P. roqueforti* isolates were chosen with the random function in Microsoft Office Excel and these selected isolates (n = 79) were screened for their secondary metabolites. All 78 *P. paneum* isolates were screened for secondary metabolite production. The isolates selected, of both species, were sourced from 102 bales on 77 farms. Isolates were maintained throughout the study on malt extract agar (MEA) plates (Oxoid, Basingstoke, UK) at 2 - 4 °C in darkness and were sub-cultured no more than six times prior to secondary metabolite analyses.

In a separate study, silage samples from three bales sourced from different farms (one bale per farm) were collected. A visually mouldy and a visually non-mouldy sample were collected from two of the bales (i.e. two samples per bale) and a fifth sample was taken from a third bale free of all visible mould growth on its surface. A representative silage grab sample (100 g) was collected in each case. The visible mould on the surface of two of these bales was recognized as *Penicillium* spp. and a small sample of mould was collected from each bale for species identification. Silage samples were stored at *ca* 4 °C while in transit and then at -18 °C until required for secondary metabolite analysis. Five grab samples of silage were taken from non-mouldy parts of each of these bales and assayed for dry matter (DM) concentration by drying (85 °C for 16 h) in a forced-air oven.

Results: The range of secondary metabolites detected in both *P. roqueforti* and *P. paneum* in this study are summarised in Tables 2.6.1 and 2.6.2, respectively. Approximately 90 % of *P. roqueforti* isolates were consistent producers of roquefortine C and andrastin A, but varied greatly in their ability to produce roquefortine A, citreoisocoumarin, andrastin C, PR toxin, eremofortin C and an unidentified metabolite. This unidentified metabolite is probably an analogue of scytalone or hydroxy-mellein and had a molecular composition of $C_{10}H_{10}O_4$ (from ESI^- LC-MS, mass deviation < 5 ppm). UV absorptions were 216 nm (100 %), 260 nm (62 %) and 334 nm (21 %). Patulin, marcfortine, gentisic acid and botryodiploidin production by *P. roqueforti* were not detected. The majority of *P. paneum* isolates were consistent producers of andrastin A, citreoisocumarin, marfortines and roquefortine C but were not consistent in their ability to produce roquefortine A, andrastin C, gentisic acid and patulin (Table 2.6.3). Lack of consistency in metabolite production was also observed in isolates collected from the same silage bale (e.g., isolates WH121E - WH121M in Table 2.6.2). Roquefortine C and andrastin A were the two most consistently produced metabolites by both species and in the case of roquefortine C, *P. roqueforti* typically produced 5 - 20 fold more than *P. paneum.* The ratio of mycophenolic acid and PR toxin production was found to vary greatly among *P. roqueforti* isolates; the mean ratio was *ca* 50:1 (range from <0.01:1 to 424:1), with more mycophenolic acid produced than PR toxin in most cases (Table 2.6.1). *P. paneum* did not produce detectable amounts of mycophenolic acid, PR toxin, botryodiploidin, metabolite T and eremofortine.

Andrastin A, mycophenolic acid, roquefortines A and C, marcfortine A and festuclavine were present in mould-contaminated silage. The recoveries (± standard deviation, three levels analysed in duplicate) of metabolites were determined to be 80±15 % for roquefortine C, 70±10 % for mycophenolic acid, 40±20 % for patulin, and 60±20 % for penicillic acid. Patulin was detected by UV at 276 nm and ESI^- as the $[M-H]^-$ ion and penicillic acid as the $[M-H]^-$ ion with an approximate limit of detection of 0.1 - 0.5 mg g-1 of silage. As sufficient quantities of the marcfortines, and roquefortines A, B, D, and OH-C (detected by ESI^+) were not available for a fortification experiment, the quantities of these were estimated from calibration curves using

pure substances, and also assuming the same recovery as for roquefortine C and a detection limit of 40 - 200 µg kg^{-1} of silage estimated from the lowest amount of the fortified sample (2 mg kg-1). Mycophenolic acid and andrastin A were detected in both polarities with a negative LC-ESI-MS being most sensitive giving a detection limit of 100 - 200 µg kg^{-1} of silage (based on the recovery level of mycophenolic acid).

Visually mouldy silage samples contained up to 20 mg kg^{-1} (74 mg kg^{-1} DM) each of roquefortine C, mycophenolic acid and andrastin A along with minor quantities (0.1 - 5 mg kg^{-1}) of the roquefortines A, B and D, festuclavine, marcfortine A and agroclavine (Table 2.6.4). Visually non-mouldy silage samples contained low amounts (<0.1 - 5 mg kg^{-1}) of mycophenolic acid and andrastin A.

Notwithstanding that the *Penicillium*-like colonies growing on bales were identified as *P. roqueforti,* the presence of marcfortine A with mycophenolic acid in three of the silage samples suggests that *P. paneum* and *P. roqueforti* were present together because *P. paneum* is the only known producer of marcfortine.

The following metabolites were not detected in any of the silage samples: verruculogen, fumitremorgin B, bis-dethio-bis(methylthio)-gliotoxin, or gliotoxin *(Aspergillus fumigatus* metabolites); citrinin *(Monascus* metabolite); patulin or marcfortine B *(P. paneum* metabolites); penicillic acid or penitrem A *(P. carneum* metabolites). The presence of patulin in feed for livestock would be a concern because it can alter metabolism of nutrients by ruminal microbes (Tapia *et al.* 2002).

This is the first report of the toxigenic potential of *P. roqueforti* and *P. paneum* growing on grass silage in Ireland. Owing to the large number of secondary metabolites produced by these two species in this and other studies, both in culture and in silage, there is a need to carry out multimycotoxin analysis on this feedstuff on a larger scale. A combination of mycotoxins and compounds not themselves being toxic rather than any one mycotoxin may be a more likely cause of livestock health problems and previous studies have not adequately investigated synergistic effects.

Conclusion: This study established the secondary metabolites produced *in vitro* by *P. roqueforti* and *P. paneum* isolated from baled grass silage in Ireland. Two factors that may affect patulin production by *P. paneum,* that is, trace metal supplements and duration of isolate storage prior to secondary metabolite screening, require further research. Secondary metabolites produced by *P. roqueforti* and also presumably *P. paneum* were detected in visually mouldy silage contaminated with *P. roqueforti* and in silage samples that did not appear to be mouldy.

Table 2.6.1. Secondary metabolites produced in culture by *P. roqueforti* (n=79 isolates) isolated from baled grass silage in Ireland.

Isolate[1, 3]	IBT[4] no..	RA[6]	RC	CIC	AA	AC	SC	EC	MPA	PR	MPA:P ratio[5]
CN100E	25410	+	+		+			+	+	+	2.70
CN102A	25416	+	+		+		+	+	+		na
CN104C	25386	+	+	+	+		+	+	+	+	45.18
CN105C$_1$	25370		+		+		+	+	+	+	6.80
CN105C$_2$	25408	+	+	+	+			+	+	+	31.40
G203B$_1$[2]	-		+	+	+	+			+	+	0.97
G203B$_2$[2]	-		+	+	+				+	+	0.72
KE10D$_2$	25376	+	+	+	+	+	+	+	+	+	6.14
KE10G	25373	+	+	+	+	+	+	+			na
KE11B	25377	+	+					+	+	+	24.53
KE11G[2]	25372	+	+	+	+		+		+		na
KE17E	25369	+	+	+	+	+	+	+			na
KE18C	25375		+		+		+	+	+	+	28.51
KE18F	25384	+	+	+	+	+	+	+		+	na
KE18G[2]	25389	+	+		+		+	+	+	+	<0.01
KE19E	25400	+	+	+	+	+	+		+	+	16.33
KE19I$_2$	-		+	+	+				+	+	117.83
KE25D	25390	+		+	+		+		+		na
KE28G[2]	25338	+	+		+	+			+		na
KE28I	25387		+					+	+	+	137.73
KE29E	25368	+	+	+	+		+	+	+	+	107.75
KE29H	25471	+	+	+	+		+	+	+	+	178.80
KE29I	25396	+	+	+	+		+	+	+	+	6.29
KK09A	25399	+	+		+		+	+	+	+	6.31
KK09B[2]	25388	+	+		+	+	+	+	+	+	47.28
LD100A	25421	+	+		+		+	+	+	+	33.55
LD100E	25422		+		+	+	+	+	+	+	9.93
LD101B	25424		+		+	+		+	+	+	37.50
LD101F	25420		+		+			+	+	+	70.71
LD102B	25412	+	+		+			+	+	+	97.55
LD103D	25411	+	+		+			+	+	+	50.50
LD104G	25409	+	+	+	+				+	+	200.00
LD104I$_2$	25401	+	+		+	+	+	+	+	+	22.07
LD105B	25406		+	+	+		+	+	+	+	209.33
LD105D	25402	+	+					+	+	+	44.59
LS01G	25391	+	+	+		+	+	+	+		na
LS04A	25392	+	+	+	+	+	+	+	+	+	10.69
LS05A[2]	25393		+	+	+		+	+			na
LS06H	25380	+	+	+				+	+	+	15.53
LS08A	25394	+	+	+	+		+	+	+	+	1.88
LS08C[2]	-		+	+	+			+	+	+	0.47
MH02D	25374	+	+	+	+		+	+	+	+	2.23

Table 2.6.1 continued on next page

Table 2.6.1 (continued). Secondary metabolites produced in culture by *P. roqueforti* (n=79 isolates) isolated from baled grass silage in Ireland.

Isolate[1, 3]	IBT[4] no.	RA[6]	RC	CIC	AA	AC	SC	EC	MPA	PR	MPA:PR ratio[5]
MH02F[2]	25371	+	+		+	+	+	+	+	+	0.02
MH03C[2]	25334	+	+	+	Trace	+	+	+	+	+	1.25
MH03G	25367	+	+		+		+				na
MH04F[2]	25337	+	+	+	+	+		+	+		na
MH07B	25470		+		+			+	+	+	99.21
MH08F	25378		+	+	+	+	+	+	+	+	85.63
MH09E	-		+		+	+			+	+	3.40
MH10A	25335	+	+		+		+	+	+	+	26.53
MH12D	25395		+	+	+	+		+	+	+	7.18
MH14C[2]	25336	+	+		+	+		+	+	+	0.45
OY100G	25418	+	+	+	+	+	+	+			na
OY1 01B	25417	+	+		+		+	+	+	+	7.07
OY107C	25404	+	+		+		+	+	+	+	38.69
OY108A	25385	+	+		+	+	+	+	+	+	45.55
OY109C	25382	+	+		+		+	+	+	+	1.33
OY1 1 1H	25381	+	+		+		+	+			na
OY1 11K	25473	+	+		+			+			na
OY113F[2]	-			+	+	+		+	+	+	1.25
OY128T_1[2]	-		+	+	+				+	+	0.01
RN209B$_2$	-		+		+			+	+	+	21.53
TN1 01 C	25419		+		+	+		+			na
TN102A	25472		+		+		+		+	+	28.40
TN103C	25429	+	+		+			+	+	+	106.90
TN106H	25398	+	+		+		+	+			na
TN106I	25426	+	+		+			+	+	+	116.30
TN107A	25403	+	+		+	+	+	+	+	+	17.95
TN107I	25405		+		+		+	+	+	+	53.83
TS12B	25379	+	+		+	+	+		+		na
TS14C	25383	+			+			+	+	+	61.95
WH100A	25414		+		+				+	+	6.31
WH100F	25413	+	+	+	+	+			+	+	424.70
WH1 01E	-	+	+		+				+	+	1.98
WH102E	25407	+	+		+	+	+	+	+	+	98.73
WH1 03B_2	25397	+	+					+	+	+	23.45
WH103B_1	25427	+	+		+	+		+			na
WH103E	25333	+	+	+		+	+	+			na
WX2 1 0A[2]	-		+		+		+	+	+	+	0.01

[1]Isolates were grown on YES and CYA media for 7 d at 25 ºC and secondary metabolites were extracted from both media and analysed using LC-UV. [2]The presence of metabolites was confirmed using LC-MS; +, denotes presence of a metabolite; na, not applicable. [3]Isolates denoted by the same two-letter code followed by an identical two- or three-digit number were collected from different points on the same bale of silage. [4]IBT, culture collection at Centre for Microbial Biotechnology, Lyngby, Denmark. [5]The peak area of mycophenolic acid was compared to PR toxin and the ratio calculated. [6]*Abbreviations:* RA, roquefortine A (isofumigaclavine A); RC, roquefortine C.; CIC, citreoisocoumarin; AA, andrastin A; AC, andrastin C; SC, scytalone or hydroxy-mellein derivative (see text); EC, eremofortin C; MPA, mycophenolic acid & analogues; PR, PR toxin.

Table 2.6.2. Secondary metabolites produced in culture by P. paneum (n=7 8 isolates) isolated from baled grass silage in Ireland

Isolate[1,2]	IBT[3]	RA[5]	RC	CIC	AA	AC	MA	PA	GA
C203A			+	+	+		+	+	+
CN100J			+	+	+		+		
CN100K	25415		Trace	+	+	+	+		
CN103C			Trace	+	+		+	+ [4]	
CN204A		+	+	+	+		+	+	
DL205A			+	+	+		+		
DL227A		+	+	+	+		+	+	
DL251J			+	+	+		+		
DL253A			+	+	+		+	+	+
G216B		+	Trace	+	+		+		
G218A		+	+	+	+		+	+	
G223A		+	+	+	+		+	+	
KE26B		+	+	+	+	+	+	+	
KE26C		+	+	+	+	+	+		
KE27D			+	+	+	+	+	+	
KE27E		+	+	+	+		+	+	
KE28B		+	+	+	+	+	+	+	
KE28C		+	+	+	+		+	+	
KE28D		+	Trace	+	+		+	+	
KE29F			Trace	+	+		+	+	
KE29G			+	+	+		+		
KK04A	25331		+	+	+	+	+	+	+
KK04B	25332	+	+	+	+		+	+	+
KK06B			+	+	+		+		
KK08B		+	+	+	+		+		
KK214B			+	+	+		+		
KY222B		+	+	+	+		+		
LD104I1	25330	+	+	+	+	+	+	+	
LD106C		+	+	+	+	+	+		+
LK207A		+	+	+	+		+		
LK210B			+	+	+	+			
LK225B			+	+	+	+	+	+	
LS06C		+	Trace	+	+		+	+	
LS06E2		+	+	+	+		+	+	
LS08E2		+	+	+	+		+	+	
MH08G		+	+	+	+		+		
MN204B		+	+	+	+		+		
MO204A		+	+	+	+	+	+	+	
OY112C		+	+	+	+		+		

Table 2.6.2 continued over/

Table 2.6.2 continued. Secondary metabolites produced in culture by *P. paneum* (n=7 8 isolates) isolated from baled grass silage in Ireland.

IIsolate[1,2]	IBT[3]	RA[5]	RC	CIC	AA	AC	MA	PA	GA
OY112H			Trace	+	+	+	+	+	
OY113A			Trace	+	+	+	+	+[4]	
OY116D			+	+	+		+		
OY118B			Trace	+	+		+	+	
OY1 18K			Trace	+	+		Trace	+	
OY122C			Trace	+	+		+	+	
OY123A			+	+	+		+		
OY127E		+	+	+	+		+	+[4]	
OY127H		+	+	+	+	+	+		
OY127I		+	+	+	+		+	+	
OY128H		+	+	+	+		+		
OY128O		+	+	+	+		+		
OY129B			+	+	+		+	+	
RN203A		+	+	+	+		+	+	
SO202A		+	+	+	+		+		
TN103A		+		+	+		+		
TN104C			Trace	+	+		+		
TS13G		+	+	+	+	+	+		
TS20G		+	+	+	+		+		
WD203A			Trace	+	+		+	+	
WD208A		+	Trace	+	+		+		
WD214A			+	+	+			+	
WD217A			+	+	+		+		
WH101C	25423		+	+	+		+		
WH105D		+	+	+	+		+	+	
WH109D			Trace	+	+		+		
WH109F		+	+	+	+		+	+	+
WH113D		+	Trace	+	+		+	+	
WH115B		+		+	+		+	+	
WH116H		+	+	+	+		+	+[4]	
WH120S			+	+	+	+	+		
WH121E			+	+	+		+		
WH121G		+	Trace	+	+		+	+	
WH121H			+	+	+		+		
WH121I		+	+	+	+		+		
WH121J			+	+	+		+		
WH121K			Trace	+	+		+		
WH121L			+	+	+	+	+		
WH121M			+	+	+		+		

[1] Isolates were grown on YES, CYA and PDA media for 7 d at 25 ºC. Secondary metabolites were extracted from the media and analysed using LC-UV and LC-MS; +, denotes presence of a metabolite. [2] Isolates denoted by the same two letter code followed by an identical two or three digit number were collected from different points on the same bale of silage. [3]IBT, culture collection at Centre for Microbial Biotechnology, Lyngby, Denmark. [4]Patulin production only detected when grown separately on Difco PDA supplemented with manganese and other trace metals. [5]*Abbreviations:* RA, roquefortine A (isofumigaclavine A); RC, roquefortine C; CIC, citreoisocoumarin; AA, andrastin A; AC, andrastin C; MA, marcfortine A; PA, patulin; GA, gentisic acid.

Table 2.6.3. Consistency of secondary metabolite production in culture by *P. roqueforti* and *P. paneum* isolates from baled grass silage in Ireland.

Secondary metabolites	Number of isolates (% of total isolates)	
	P. roqueforti (n = 79)	*P. paneum* (n = 78)
Mycophenolic acid & analogues	67 (84.8)	ND
Roquefortine A	55 (69.6)	42 (53.8)
Roquefortine C	76 (96.2)	76 (97.4)
Marcfortines	ND	76 (97.4)
Citreoisocoumarin	33 (41.7)	78 (100)
Andrastin A	72 (91.1)	78 (100)
Andrastin C	30 (38.0)	17(21.8)
Metabolites T	45 (57.0)	ND
PR toxin	61(77.2)	ND
Eremofortin C	63 (79.7)	ND
Patulin	ND	39 (50)
Gentisic acid	ND	6(7.7)

ND, not detected

Table 2.6.4. Secondary metabolites detected in fresh samples of grass silage from bales in Ireland

Sample no.	FC[4]	AC	RA	RB	OHRC	RC	RD	MA	CIC	MPA	AA
1[1]	++	++	+++	++	++	++++	++	++	tr	++++	++++
2[1]	++	++	+++	++	ND	++++	++	++	tr	++++	++++
3[2]	ND	ND	ND	ND	ND	ND	ND	ND	ND	tr	++
4[2]	ND	ND	ND	ND	ND	ND	ND	+	ND	+++	++
5[3]	ND	ND	ND	ND	ND	ND	ND	ND	ND	tr	++

[1]Visually mouldy silage (fungus identified as P. roqueforti). 2Visually non-mouldy silage adjacent to mold colonies on the same bale. 3Visually non-mouldy silage from a bale free of all visible mould growth on its surface. Bale 1, samples 1 and 3 (Dry matter (DM) = 27.2 %); Bale 2, samples 2 and 4 (DM = 44.8 %); bale 3, sample 5 (DM = 51.9 %); ++++, 5 to 20 mg kg-1 range; +++, 1 to 5 mg kg-1 range; ++, 0.1 to 1 mg kg-1 range; +, < 0.1 mg kg-1. Metabolites were detected by LC-ESI+-MS, except CIC, which was only detected by LC-ESI—MS. MPA and AA were detected in both polarities with LC-ESI—MS being the most sensitive. ND not detected, which equals < 40-200 µg kg-1 of all metabolites except CIC. Trace, peak detected (s/n <10); 4Abbreviations: FC, festuclavine; AC, agroclavine; RA, roquefortine A, RB, roquefortine B; RC, roquefortine C; OHRC, 1 6-hydroxyroquefortine C; RD, roquefortine D; MA, marcfortine A; CIC, citreoisocuomarin; MPA, mycophenolic acid; AA, andrastin A.

Experiement 2.7: Morphological and molecular characterisation of *Penicillium roqueforti* and *Penicillium paneum* isolated from baled grass silage in Ireland

[M. O'Brien, Egan, D., O'Kiely, P., Forristal, P.D., Fuller, H.T., Doohan, F.M.]

This study aimed to characterise the morphological, cultural and molecular characters of *P. roqueforti* and *P. paneum* isolated exclusively from baled grass silage in Ireland. Their molecular characterisation was based on the partial sequences of β tubulin and acetyl CoA synthetase genes.

Materials and methods: *Sample collection and isolate selection:* The incidence of fungal growth on baled grass silage (*n* = 464 bales) on Irish farms (*n* = 235 farms) was recorded in three of the previous experiments. A total of 2277 visible fungal colonies were enumerated on these bales and 1190 fungal colonies were sampled and cultured. Isolates were maintained throughout the study on malt extract agar (MEA) plates (Oxoid, Basingstoke, UK) at 2 - 4 °C, in darkness. Of the fungal isolates, 830 were identified as *P. roqueforti* and 78 as *P. paneum* by their macro- and micromorphological features; results were confirmed based on LC-UV and LC-MS analysis of secondary metabolites produced by both fungi.

A subset of the *P. roqueforti* isolates (*n* = 237) were randomly selected and their macromorphological features were examined in more detail. Subgroups of these isolates were randomly selected for micromophological (*n* = 38) and molecular analyses (*n* = 38). In the case of *P. paneum*, the macromorphological features of all 78 isolates were examined and the micromorphological features and molecular characteristics of randomly chosen subsets of isolates (*n* = 20 and 15, respectively) were analysed. These fungal isolates selected for morphological and molecular characterisation were thus sourced from 119 bales that were obtained from 93 farms country-wide.

Results: *Macromorphology features of P. roqueforti and P. paneum:* For both *Penicillium* species, colonies growing on CYA and YES (and MEA for *P. paneum*) were normally olive to olive brown coloured in their centre (Tables 2.7.1 and 2.7.2), an observation not recorded previously.

Several differences between *P. roqueforti* and *P. paneum* emerged in this study, perhaps reflecting the large numbers of isolates examined for each species. *P. paneum* isolates grew faster on all three media (i.e. CYA, YES and MEA) and colony colours on CYA and YES were a darker shade of green than were those of *P. roqueforti*. *P. roqueforti* colony reverse on CYA was a distinctive dark green to black colour after 7 days incubation compared to the intense orange brown observed for *P. paneum*. Another difference between species on CYA medium was the ability of *ca* 50 % of the *P. paneum* isolates to produce exudate droplets; none of the *P. roqueforti* isolates exhibited this characteristic on CYA.

On MEA, colony colour was dull green or greyish green for *P. roqueforti* and jade green for *P. paneum*. Growth at 5 and 37 °C were similar for both species, and this concurred with the literature. The ability of isolates to grow at low a_w was assessed with G25N, which has a reduced water activity of *ca* 0.93. *P. roqueforti* was found to grow *ca* 25 % quicker than *P. paneum* after 7 days on this medium and this has not been reported previously in the literature. The reverse coloration of *P. paneum* cultures on YES was beige to blond with no pigments diffusing into the medium.

Micromorphological features of P. roqueforti and P. paneum: Both *P. roqueforti* and *P. paneum* were primarily terverticillate, with rough stipes, ampulliform phialides and globose conidia (Tables 2.7.3 and 2.7.4, respectively). The micromorphological differences between species included *P. paneum* having larger conidiophore structures (i.e. stipes, rami, metulae), phialides and conidia than *P. roqueforti*. Although the conidiophores, phialides and conidia were within the ranges listed in the literature for both species, *P. roqueforti* was at the lower end, whereas *P. paneum* was at the mid to high end of these ranges.

Prior to this study, both species were regarded as having smooth-walled conidia. SEMs of conidia from both *P. roqueforti* and *P. paneum* isolates collected in this study show that the conidia have a finely rough surface texture. *P. paneum* conidia had a rougher surface texture than *P. roqueforti*. Under light microscopy, all *P. paneum* conidia examined were observed to be finely rough surfaced, whereas only 28 % of *P. roqueforti* conidia were observed to be finely rough. The stipe texture of both species was quite variable. In *P. roqueforti*, 8 % of isolates examined had predominantly smooth stipes, 8 % were finely rough, 68 % were rough, 11 % were very rough and 3 % were tuberculate. In the case of *P. paneum*, 30 % of isolates had predominantly rough stipes, while the remaining 70 % had very rough stipes.

Phylogenetic analysis: The primers acuA-2F and acuA-1R used in this study amplified 282-bp (*P. roqueforti*) and 290-bp (*P. paneum*) fragments spanning introns 3 and 4. BLASTn analysis of the β tubulin sequences identified *P. roqueforti* and *P. paneum* as the closest homologs of our *P. roqueforti and P. paneum* isolates, respectively (99 – 100% homology; results not shown). There were no acetyl CoA synthetase sequences available in GenBank for either *P. roqueforti* or *P. paneum*, so no comparisons could be made. Alignment (using ClustalW) showed that the sequences

of the β tubulin and acetyl CoA synthetase gene fragments were highly conserved within the two *Penicillium* species used in this study. The partial β tubulin sequence distinguished the 38 *P. roqueforti* isolates examined into three groups.

The partial acetyl CoA synthetase sequence also distinguished three groups of *P. roqueforti* isolates; 35 isolates comprised group 1 which differed in two bp from two isolates that comprised group 2 and from one isolate that comprised group 3. There was no variation in the sequence of the β tubulin and acetyl CoA synthetase gene fragments between the 15 *P. paneum* isolates. The *P. paneum* β tubulin sequences were identical sequences to two of the three published *P. paneum* sequences available in GenBank (AY674387 and AY674389; isolated from ryebread) and differed in a single nucleotide deletion from an isolate originating from mouldy baker's yeast (AY674388).

Most parsimonious trees (MPT) generated from individual gene datasets showed compatible topologies, supporting the analysis of these datasets in combination (data not shown). Combined analysis of data from the partial β tubulin and acetyl CoA synthetase sequences included 53 taxa comprising 38 *P. roqueforti* and 15 *P. paneum*. An exhaustive search of the combined dataset (728 bp, 117 parsimony-informative characters) produced 23 MPT (CI and RI of 0.883 and 0.979, respectively). NJ analysis supported the overall topology from the MP analysis and a phylogenetic tree was produced. The two species in this study were well supported by bootstrap (100 %). *P. paneum* strains were monophyletic and variation within *P. roqueforti* isolates did not receive strong bootstrap support (52 %). The fact that all isolates were sourced from a common substratum in an island geographical region may explain the lack of variability within each species.

Conclusions: This is the first significant record of the morphological, cultural and molecular characteristics of *P. roqueforti* and *P. paneum* isolates from grass silage. Considering the importance of grass silage as a feed source for livestock, this definitive description of two common spoilage and toxigenic moulds will greatly help other investigators to arrive at a proper identification.

Table 2.7.1 - Macromorphological characteristics of *Penicillium roqueforti s.s.* isolated from baled grass silage in Ireland and comparisons with characteristics reported in the literature for *P. roqueforti s.s.* and *s.l.* sourced from a wide range of substrates and geographical locations

Character	Present study (*n*=237 isolates)[a, b]	Literature[b, c]
CYA colony:		
Diam (mm)	(11-) 40 (-70)	(17-) 40 (-77)
Colour	Olive brown (centre) to dull green[d]	Green
Reverse	Dark green (to black[e])	Blackish green
Colony texture	Velutinous	Velutinous
Medium buckling	Absent	Radially sulcate[f]
Colony margins	Arachnoid	Arachnoid[g]
Exudate droplets on colony	Absent	Absent
Diffusable colours	Absent	Absent
YES colony:		
Diam (mm)	(30-) 54 (-72)	38 - 61
Colour	Olive (centre) to dull green	Green[g]
Reverse	Dull green to dark green (to black[e])	Blackish green
Colony texture	Velutinous	ND
Medium buckling	Wrinkled	Wrinkled[h]
Colony margins	Entire	ND
Exudate droplets on colony	Absent	ND
Diffusable colours	Absent	ND
MEA colony:		
Diam (mm)	(27-) 50 (-71)	26 – 43
Colour	Dull green[d]	Dull green[f]
Reverse	Beige to greyish green	Pale to brown to black[f]
Colony margins	Arachnoid	Arachnoid[g]
CYA @ 5 °C (diam, mm)	(0-) 4 (-11)	2 – 4
CYA @ 37 °C (diam, mm)	No growth	No growth
G25N (diam, mm)	(7-) 20 (-25)	20 – 22 (-28)[f]
Growth on 0.5% acetic acid	Yes	Yes

[a] Observations were recorded after incubation for 7 d at 25 °C, unless otherwise stated; [b]Measurements are presented as means with extremes in brackets; [c]Primarily adapted from Frisvad & Samson (2004) unless stated otherwise; ND = not described; [d]*ca.* 10 % of isolates were distinctively olive brown to greyish green; [e]Observation recorded after incubation for 14 d at 25 °C; [f]From Pitt (2000); [g]From Boysen *et al.* (1996); [h]From photograph (in Frisvad & Samson 2004).

Table 2.7.2 - Macromorphological characteristics of *Penicillium paneum* isolated from baled grass silage in Ireland and comparisons with characteristics reported in the literature for *P. paneum* sourced from a wide range of substrates and geographical locations

Character	Present study (*n*=78 isolates)[a, b]	Literature[c]
CYA colony:		
Diam (mm)	(30-) 48 (-60)	38 - 41
Colour	Olive brown (centre) to dull green to dark green	Blue green to green
Reverse	Greyish orange (to brownish orange[d])	Beige to brown
Colony texture	Velutinous	Velutinous
Medium buckling	Irregular wrinkling	ND
Colony margins	Arachnoid to entire	Entire[e]
Exudate droplets on colony	Clear to olive brown (*ca* 50% of isolates)	Copious, clear
Diffusable colours	Absent	Absent
YES colony:		
Diam (mm)	(44-) 60 (-73)	52 - 71
Colour	Olive brown (centre) to dull green to dark green	Bluish-grey-green[e]
Reverse	Beige to blond (to yellowish grey[d])	Cream yellow/beige[f]
Colony texture	Velutinous	ND
Medium buckling	Wrinkled	Wrinkled
Colony margins	Entire	Entire[e]
Exudate droplets on colony	Absent	ND
Diffusable colours	Absent	ND
MEA colony:		
Diam (mm)	(39-) 56 (-69)	43 – 67
Colour	Olive to jade green	Green[g]
Reverse	Beige to greyish green	ND
Colony margins	Arachnoid	Entire[e]
CYA @ 5 °C (diam., mm)	(0-) 2 (-7)	2 – 4
CYA @ 37 °C (diam., mm)	No growth	No growth
G25N (diam., mm)	(0-) 15 (-27)	ND
Growth on 0.5% acetic acid	Yes	Yes

[a]Observations were recorded after incubation for 7 d at 25 °C, unless otherwise stated; [b]Measurements are presented as means with extremes in brackets; [c]Primarily adapted from Frisvad & Samson (2004) unless stated otherwise; ND = not described; [d]Observation recorded after incubation for 14 d at 25 °C; [e]From Boysen *et al.* (1996); [f]Often turns to strawberry red with age and the colour diffuses into the medium (after Frisvad & Samson 2004); [g]From Boysen (1999).

Table 2.7.3 - Micromorphological characteristics of *Penicillium roqueforti s.s.* isolated from baled grass silage in Ireland and comparisons with characteristics reported in the literature for *P. roqueforti s.s.* and *s.l.* sourced from a wide range of substrates and geographical locations

Character	Present study (*n*=38 isolates)[a]	Literature[b]
Conidiophore:		
Branching pattern	Ter- , occassionly bi- or quater-verticillate	Ter- , occassionly quater-verticillate
Stipe texture	Rough	Rough
Length (µm)	(5-) 94 (-252)	100 - 250
Rami:		
Length (µm)	(6-) 16 (-40)	17 - 33
Texture	Rough	Rough[c]
Metulae:		
Length (µm)	(9-) 11 (-15)	10 - 17
Phialides:		
Length (µm)	(6-) 9 (-11)	8 - 10
Type	Ampulliform (cylindrical)	Cylindrical
Conidia:		
Shape	Globose	Globose
Colour	Greyish green	Green[d]
Texture	Smooth (72 %), finely rough (28 %)	Smooth
Size (µm)	(2-) 3.5 (-6)	(2.5-) 3.5 – 5 (-6)[e]

[a]Observations were recorded after incubation on MEA for 7 d at 25 °C. Structures were examined using light microscopy (400 - 1000x magnification). Measurements are presented as means with extremes in brackets; [b]Primarily adapted from Frisvad & Samson (2004) unless stated otherwise; [c]From Pitt (2000); [d]Conidia taken from CYA; [e]Extreme values reported by Shimada & Ichinoe (1998).

Table 2.7.4 - Micromorphological characteristics of *Penicillium paneum* isolated from baled grass silage in Ireland and comparisons with characteristics reported in the literature for *P. paneum* sourced from a wide range of substrates and geographical locations

Character	Present study (*n*=20 isolates)[a]	Literature[b]
Conidiophore:		
Branching pattern	Ter- , occassionly bi- or quater-verticillate	Ter-, occassionly quater-verticillate
Stipe texture	Rough	Rough
length (µm)	(17-) 134 (-336)	100 - 250
Rami:		
Length (µm)	(6-) 18 (-42)	17 - 33
Texture	Rough	ND
Metulae:		
Length (µm)	(9-) 14 (-20)	10 - 17
Phialides:		
Length (µm)	(8-) 10 (-13)	8 - 10
Type	Ampulliform (cylindrical)	Cylindrical
Conidia:		
Shape	Globose	Globose
Colour	Greyish green	Blue green to green[c]
Texture	Smooth (8 %), Finely rough (92 %)	Smooth
Size (µm)	(2.3-) 4.1 (-4.9)	3.5 - 5

[a]Observations were recorded after incubation on MEA for 7 d at 25 °C. Structures were examined using light microscopy (400 - 1000x magnification). Measurements are presented as means with extremes in brackets; [b]From Frisvad and Samson (2004); ND = not described; [c]Conidia taken from CYA.

3. Understanding the fermentation dynamics in baled silage

Specifically, the aims of this part of the project were:

1. Identify the factors regulating fermentation in baled silage, and quantify their relative contribution (relative to the effects in conventional silage)
2. Define how to manipulate baled silage fermentation so as to create conditions more inhibitory to undesirable micro-organisms

Experiment 3.1: The microbiological and chemical composition of baled and precision-chop silages on a sample of farms in County Meath

[McEniry, J., O'Kiely, P, Clipson, N.J.W., Forristal, P.D. and Doyle, E.M.]

The purpose of this study was to determine the microbiological and chemical composition of baled and precision-chop silages at feedout on a sample of farms. Furthermore, the impact on these parameters within baled silage was examined in the outer layer close to the plastic wrap and compared to that in the centre of the bale.

Materials and Methods: Two bales of silage were sampled on each of ten farms during February and March of both 2004 and 2005 (i.e. 20 bales per year). Also in February 2005, twenty conventional precision-chop silages were sampled. Farms were randomly selected and were located within a 10 km radius of Grange.

On each farm, the characteristics of the bale storage environment and the physical appearance of the bales and horizontal silos were recorded. Physical damage, if any, to the plastic wrap or cover and apparent causes were noted. A questionnaire detailing the history of each silage (e.g. cut date, weather at harvesting) was also completed with the aid of the farmer.

Two bales on each farm were selected on the basis of being the next in line for feeding. Bales (1.22 m wide x 1.22 m diameter) were removed for ease of sampling and the wrap was examined and damage (if any) noted. The plastic wrap was then removed. The bales of silage were aseptically sampled using an electrically powered cylindrical core bit (length = 65.0 cm; internal diameter = 3.5 cm) at eight points around the bale. Sampling points were adjacent to the clock positions 0300, 0600, 0900 and 1200 h on the bale barrel, *ca.* 40 cm from each end, where there was no visible evidence of aerobic deterioration (i.e. no visible mould or yeast growth). At each point sub-samples were taken from both the outer 20 cm and then through to the centre of the bale (inner 40 cm). The eight outer and eight inner core sub-samples were composited to produce a single outer and a single inner sample per bale. Each sample was stored at 4 ºC prior to sub-sampling for microbiological analyses and then at –18 °C for subsequent chemical analyses.

Precision-chop silage was aseptically sampled using a manual cylindrical corer (internal diameter = 3.0 cm). A single full-depth core sample was taken at the centre of the top surface of each silo, from the top layer through to the base of the silage. The sample was taken from an area where there was no visible evidence of damage to the plastic cover, and no visible surface waste or mould. These core samples were stored at 4 °C prior to sub-sampling for microbiological analyses and then at –18 °C for subsequent chemical analyses.

Results: All of the bales sampled were made during the summer of the previous year, with the majority of bales being made in June (0.50) followed by August (0.20), May (0.10) and July (0.10). Only one farm wrapped bales directly after mowing. A wilting period of 1-day was employed for proportionally 0.55 of the bales, with 0.20 of the bales being made after a 2-day wilt and the remainder after a period greater than 2-days. All bales were wrapped in black plastic stretch-film, with four layers being the most common rate applied (0.75) with the remaining farms nominally using 6 layers. Just over half the bales (0.55) were wrapped at the site of baling prior to transfer to their storage location with the remainder transported to the storage area before wrapping. All bales were fenced off from livestock, generally being stored in single tiers beside farm buildings, on storage surfaces of concrete, grass or gravel. The plastic film was visibly damaged on 0.48 of the bales, with some visible mould growth present on 0.78 of the bales.

The mean (s.d.) dimensions of the precision-chop silage silos were 25.9 m (6.42) long, 10.4 m (4.98) wide and 3.2 m (1.39) high. Precision-chop silage was made earlier in the season than baled silage, with proportionately 0.4 of the silages being made in May and 0.3 in June. Wilting was not as important in this system, with 0.7 of the herbage being ensiled on the day of mowing. The silages were generally sealed beneath polythene sheeting on the day after filling. On all farms, silages were covered with two sheets of black 0.125 mm polyethylene and weighted with tyres, although on 0.3 farms bales of straw and dung were placed over the plastic on parts of the silo.

The DM concentration of the bales (Table 3.1.1) indicates that wilting conditions were generally satisfactory and that the 1 to 2 day wilt was of sufficient duration in relation to target recommendations for Irish conditions. In contrast, precision-chop silage DM concentration reflects

a conservation process with a minimal effective wilt. The higher DM concentration in baled silage was reflected in a more restricted fermentation, with a lower ($P<0.001$) buffering capacity and content of fermentation products, and a higher ($P<0.001$) final pH compared to precision-chop silage.

In the present study, lactic acid was the dominant fermentation product in precision-chop silage, followed by acetic acid and ethanol, and with smaller amounts of propionic acid (Table 3.1.1). The contents of the fermentation products and the pH were indicative of a satisfactory preservation. The fermentation products in baled silage were less dominated ($P<0.05$) by lactic acid and conditions tended to be less inhibitory to the formation of butyric acid than in precision-chop silage. However, the extent of breakdown of protein to ammonia was adequately and similarly restricted ($P>0.05$) for both silage types.

Despite the difference ($P<0.001$) in mean lactic acid content between the two types of silage, the number of lactic acid bacteria did not differ ($P>0.05$). This suggests that irrespective of potentially different bacterial growth profiles or relative contributions of constituent species of lactic acid bacteria during ensilage, the inhibitory factors associated with silage DM and pH combined to create similar final inhibition. Such inhibition of lactic acid bacteria toward the end of ensilage is mediated through the combined effects of anaerobiosis, the presence of undissociated organic acids and water activity. The implication of a greater contribution of homofermentative rather than heterofermentative lactic acid bacteria in precision-chop silage compared with baled silage is difficult to confirm using fermentation products as a sole guide. This is because of the ability of other bacteria and/or yeast to produce acetic acid and ethanol and because some respiration of lactic acid can occur if oxygen ingress takes place during ensilage.

Despite the higher ($P<0.001$) content of WSC in the baled silage being conducive to yeast fermentation activity, the absence of considerably higher concentrations of ethanol suggest that the higher ($P<0.01$) yeast numbers in baled silage reflect less strictly anaerobic conditions during the storage of silage in bales compared with conventional horizontal silos. Besides heterofermentative lactic acid bacteria, the contribution of other bacteria to ethanol production in silage is small. Furthermore, the thinness of the stretch film barrier surrounding bales and the ease with which it can be damaged, together with the increased porosity of the wilted forage, may facilitate the passage of oxygen into and through the bale. The higher pH and lower levels of fermentation products in baled silage together with the restricted activity of lactic acid bacteria would also facilitate yeast development. The higher ($P<0.05$) numbers of clostridial spores in baled silage compared to precision-chop silage is surprising given the negative effects of a high DM concentration and of oxygen on clostridial activity. Clostridial spores can increase during aerobic deterioration, and oxygen ingress is likely to be prevented less by the plastic barrier surrounding bales than by that placed on horizontal silos. In addition to the above, the higher pH in baled silage would have been less inhibitory to clostridial activity than the mean pH in precision-chop silage. The trend towards a higher content of butyric acid in baled silage, in the absence of an elevation in ammonia-N content, suggests that clostridial activity was dominated by saccharolytic rather than proteolytic bacteria.

Numbers of *Bacillus* spores were higher ($P<0.01$) in precision-chop silage than baled silage. The lower number of spores in baled compared to precision-chop silage suggests less contamination of harvested silage by soil or animal manure. Such a difference could be related to differences in land and sward type, soil conditions at harvest, to manure spreading practices between the two silage harvesting systems or due to differences in crop mowing, handling or harvesting techniques.

In the present study, *Enterobacteria* numbers were moderately low in both types of silage prior to feedout. This suggests that a relatively rapid decrease in pH occurred with baled and precision-chop forages during ensilage and agrees with the overall finding of a satisfactory preservation. The low ammonia-N content also agrees with this interpretation, as *Enterobacteria* can have a significant role during ensilage in protein degradation and in reducing nitrate to ammonia under certain conditions.

The absence of a difference ($P>0.05$) in *in-vitro* DMD between baled and precision-chop silages agrees with the similar NDF and ADF values for both silages.

In general, the extent and pattern of fermentation (as indicated by fermentation indices prior to feedout) suggest little difference between the outer and inner layers of baled silage (Table 3.1.2). The content of the major fermentation acids and ammonia-N, the relativity of the fermentation acids to one another, buffering capacity and the content of residual WSC were similar for the two sections of the bales. This suggests a similar impact in each bale horizon by the factors controlling fermentation, giving a satisfactory overall preservation. However, the microbial composition is different between the outer and inner layers. While there was no difference ($P>0.05$) in the lactic and acetic acid content between the two bale horizons, numbers of lactic acid bacteria were significantly higher ($P<0.01$) in the outer, less anaerobic layer. This suggests that the final

inhibition within bales is not as strong in the outer layer close to the plastic wrap.
Yeast numbers ($P<0.05$) were also higher in the outer bale layer. However, higher yeast numbers were not mirrored in higher levels of ethanol, with ethanol content being greater ($P<0.05$) in the inner layer. This would suggest yeast respiration rather than fermentation in the more aerobic environment closer to the plastic wrap. This was supported further by increased numbers of *Enterobacteria* ($P<0.05$) in the outer bale layer. While *Enterobacteria* numbers were relatively low, pH values and ammonia-N content were similar in both sections, suggesting that anaerobic conditions were less exacting in the outer layer of baled silage. In contrast, higher ($P<0.05$) numbers of clostridial spores were found in the inner compared with the outer layer. The numerically modest increase in clostridial spores in the inner layer was not supported by an increase in levels of either butyric acid or ammonia-N.
Indices of silage nutritive value such as fibre, protein, WSC and ash were quite similar in both parts of the bales. However, silage digestibility was found to be lower ($P<0.05$) in the outer bale layer. Since there were no significant differences between the different bale layers in any other index of nutritive value, the lower DMD in the outer horizon may reflect a qualitative loss due to respiration, involving the possible production of Maillard products.
Conclusions: This paper describes the composition of two different sample populations of silage, produced on different farms and with different grass crops. The baled silage production and storage characteristics, and indices of nutritive value and preservation (i.e. DM, DMD, crude protein and pH) in both baled and precision-chop silages were similar to those previously reported in national surveys. Baled silages underwent a more restricted fermentation than precision-chop silages, due mainly to their higher DM concentration. However, the differences in microbiological composition between the two silage types likely reflect somewhat less anaerobic conditions within baled silage. The relatively modest differences between the outer and inner sections of baled silage suggests that generally similar ensiling conditions prevailed in both horizons, albeit with slightly less anaerobic conditions closer to the bale surface. However, sampling to a depth of 20 cm may have masked the effect of proximity to the plastic stretch-film. A more localised layered effect may have taken place closer to the bale surface (e.g. 1 cm) but this may not have had a measurable effect on overall mean values. Also, had baled silage been sampled from points on the bale where visible mould growth was present, the differences between both baled silage and precision-chop silages, and between the outer and inner horizon of bales, would have been more pronounced.

Table 3.1.1: Composition of baled (combined weighted mean) and conventional precision-chop silages

	Baled silage	Precision-chop silage	s.e.d.	Sig.
Lactic acid bacteria (log_{10} cfu/g silage)	5.70	5.96	0.205	NS
Yeast (log_{10} cfu/g silage)	3.81	2.34	0.514	**
Clostridia (log_{10} cfu/g silage)	3.70	3.04	0.308	*
Bacilli (log_{10} cfu/g silage)	2.67	3.46	0.275	**
Enterobacteria (log_{10} cfu/g silage)	1.27	1.31	0.498	NS
Dry matter (g/kg)	360	220	26.3	***
pH	4.55	3.85	0.115	***
Lactic acid (g/kg DM)	42	103	8.1	***
Ethanol (g/kg DM)	15	19	2.1	*
Acetic acid (g/kg DM)	15	43	2.9	***
Propionic acid (g/kg DM)	3.2	7.0	0.78	***
Butyric acid (g/kg DM)	10.4	6.2	2.14	NS
Volatile fatty acids (g/kg DM)	28	57	4.3	***
Fermentation products (g/kg DM)$^{\$}$	85	179	9.2	***
Lactic acid/fermentation products (g/g)	0.49	0.58	0.046	*
NH_3-N (g/kg N)	69	85	8.8	NS
Dry matter digestibility (g/kg)	644	677	20.5	NS
Neutral detergent fibre (g/kg DM)	547	548	12.9	NS
Acid detergent fibre (g/kg DM)	322	340	7.3	NS
Ash (g/kg DM)	93	96	4.6	NS
Crude protein (g/kg DM)	135	156	6.7	**
Buffering capacity (m. Eq/kg DM)	562	822	32.5	***
Water soluble carbohydrate (g/kg DM)	62	11	6.5	***

* = P<0.05, ** = P<0.01, *** = P<0.001, NS = not significant, cfu = colony forming unit,
$^{\$}$ Fermentation products = lactic acid, acetic acid, propionic acid, butyric acid and ethanol

Table 3.1.2: Composition of the outer and inner layers of baled silage (n = 40 bales)

	Bale outer	Bale inner	s.e.d.	Sig.
Lactic acid bacteria (log_{10} cfu/g silage)	5.83	5.55	0.102	**
Yeast (log_{10} cfu/g silage)	4.07	3.50	0.213	*
Clostridia (log_{10} cfu/g silage)	3.56	3.86	0.123	*
Bacilli (log_{10} cfu/g silage)	2.77	2.57	0.117	NS
Enterobacteria (log_{10} cfu/g silage)	1.44	1.07	0.173	*
Dry matter (g/kg)	359	362	9.0	NS
pH	4.54	4.55	0.030	NS
Lactic acid (g/kg DM)	42	42	1.8	NS
Ethanol (g/kg DM)	14	17	1.0	*
Acetic acid (g/kg DM)	15	15	1.2	NS
Propionic acid (g/kg DM)	2.8	3.7	0.53	NS
Butyric acid (g/kg DM)	10.3	10.6	0.76	NS
Volatile fatty acids (g/kg DM)	28	29	1.8	NS
Fermentation products (g/kg DM)$^{\$}$	84	88	3.4	NS
Lactic acid/fermentation products (g/g)	0.50	0.48	0.011	NS
NH_3-N (g/kg N)	71	67	4.5	NS
Dry matter digestibility (g/kg)	636	653	7.0	*
Neutral detergent fibre (g/kg DM)	552	541	5.8	NS
Acid detergent fibre (g/kg DM)	325	319	5.6	NS
Ash (g/kg DM)	95	92	2.6	NS
Crude protein (g/kg DM)	137	132	4.4	NS
Buffering capacity (m. Eq/kg DM)	559	565	14.1	NS
Water soluble carbohydrate (g/kg DM)	62	63	4.8	NS

* = P<0.05, **= P<0.01, NS = not significant, cfu = colony forming unit,

$^{\$}$ Fermentation products = lactic acid, acetic acid, propionic acid, butyric acid and ethanol

Experiment 3.2. The microbiological and chemical composition of silage over the course of fermentation in round bales relative to that of precision-chop silage

[J. McEniry, P. O'Kiely, N. J. W. Clipson, P. D. Forristal and E. M. Doyle]

Experimentally it is difficult to compare baled and precision-chop silages with adequate replication on a farm scale. This study firstly aimed to compare unchopped herbage in bales and laboratory silos to determine if laboratory silos were a reasonable model for baled silage fermentation. A previous study confirmed the relationship between precision-chop silage made in laboratory silos and small farm silos. The main aim of the experiment was to compare unchopped and chopped grass ensiled in laboratory silos, as representative systems for baled and precision-chop silages, respectively. These comparisons were replicated at three DM concentrations so as to quantify interactions of herbage DM concentration with conservation system. The final aim was to quantify the dynamics of the fermentation within the various treatments. This information should contribute to understanding why the conservation characteristics of individually wrapped bales of unchopped silages often differ from those of other more conventional silages.

Materials and methods: The experiment was organised in a 3 (duration of wilt treatments) x 3 (ensiling system) x 6 (stage of ensiling) factorial arrangement of treatments. Replicated plots of herbage were wilted for 0, 24 and 48 h, and in each case representative samples were ensiled unchopped in large cylindrical bales, and unchopped or precision-chopped in laboratory silos. Three bales and three of each of the laboratory silo chop treatments were destructively sampled after 2, 6, 14, 35 and 98 days ensilage (as well as herbage being sampled immediately prior to ensiling). The microorganisms of significance were enumerated and fermentation variables were assessed at each sampling time. Silage aerobic stability was assessed after 98 days ensilage.

Herbages were produced and ensiled at Grange. An homogenous plot of *Lolium perenne* (cv. Fennema) was mown (Pottinger, Nova 310T conditioner mower) on the 18 May 2004 and field wilted for up to 48 h. There was no rainfall during harvesting and drying conditions were considered excellent. After 0, 24 and 48 h wilting, representative samples of herbage from throughout the crop were picked up with a round baler (Claas 250 Rotacut) and baled into fifteen 1.2 m wide x 1.2 m diameter cylindrical bales. These bales were labelled, weighed and wrapped (McHale, 991BE) in 6 layers of polythene film (750 mm wide, 0.025 mm thickness pre-stretching) with a 1.70 pre-stretch applied. The wrapped bales were placed gently on their flat ends on a bed of fine sand in an order that would facilitate representative sampling of all the herbage at each sampling date. They were fenced off from livestock, protected by bird netting and the area was baited for rodents. Each bale was allowed to stand in individual isolation until opening.

After each group of fifteen bales were removed from the field, the remaining herbage was tedded (Krone rotary tedder, KW550/4x7) throughout the afternoon and was rowed (Krone windrower, KS380) up in the late evening.

At each time of baling, 30 representative samples of herbage were obtained from across the field for ensiling in laboratory silos. On each occasion, half of these samples were precision-chopped (Pottinger, Mex VI) immediately prior to ensiling. The chopping knife number and feed roller speeds were chosen, according to the manufacturers instructions, to give a theoretical chop length of 19 mm. The remaining 15 silos were filled with unchopped herbage. Each laboratory silo was filled with a similar quantity of herbage which resulted in weights of 5, 4.7 and 4.1 kg herbage, respectively, corresponding to the 0, 24 and 48 h wilt treatments. The silos (height = 0.75 m, internal diameter = 0.152 m, internal volume = 13.6 l) were packed manually and sealed immediately by a screw-on top with a rubber seal. Compaction was achieved in the silos by the inclusion of a 10.5 kg weight (diameter = 0.1 m) directly on the herbage to exert continual vertical pressure (5.83 kPa). The 90 laboratory silos were stored at 15ºC prior to opening.

After 0, 24 and 48 h wilting, triplicate representative samples were taken to characterise the grass at time 0.

At each herbage DM concentration, triplicate bales and unchopped and chopped laboratory silages were destructively sampled after 2, 6, 14, 35 and 98 days ensilage. The bales were checked for damage to the surrounding plastic wrap, weighed and aseptically sampled, through to the centre, at eight points around the bale using an electrically powered aseptic cylindrical core bit (length = 65.0 cm; internal diameter = 3.5 cm). Sampling points were at 0300, 0600, 0900 and 1200 h clock positions on the bale barrel, *ca.* 40 cm from each end. These eight core samples were composited to produce one sample per bale.

Silage from the laboratory silos was weighed and sampled. Effluent from the silos (if any) was also collected and weighed. After thorough aseptic mixing, one sample per silo was taken.

Grass and silage samples were stored at 4°C prior to microbiological analyses and at –18°C prior to chemical analyses.

Once each silage was sampled on day 98, the remainder of the herbage from the laboratory silos was

used to assess for aerobic stability and deterioration. Silage bales were split in two, aseptically mixed and representatively sampled to provide an equal quantity of baled silage for assessment as used from the laboratory silos. Aerobic stability assessments were made using 3.4, 3.1 and 2.7 kg silage for each replicate from the 0, 24 and 48 h wilted treatments, respectively.

Results: Due to the large amount of data generated, much of the data presented represents the main factor interactions between wilt and stage of ensiling, and ensiling system and stage of ensiling. Other factor interactions are presented only if statistically significant.

Mean (s.d.) bale weights were 867 (18.9), 828 (40.7) and 725 (50.7) kg for the 0, 24 and 48 h wilt treatments at ensiling, respectively. There was no damage to the plastic film on any of the bales sampled. Silage DM concentration and pH increased (P<0.001; Tables 3.2.1 and 3.2.3), while silage buffering capacity and ammonia-N, lactic acid, acetic acid, propionic acid and ethanol concentrations all decreased (P<0.001) with increasing duration of wilt (Figures 3.2.1a – 1h). Butyric acid concentration was low in all silages, with a decrease (P<0.001) in concentration observed after a 48 h wilt only (Figure 3.2.1e). On average, the NDFom concentration increased (P<0.001) with increasing duration of wilt (Table 3.2.3). The numbers of Clostridia (P<0.001) and Enterobacteria (P<0.05) were higher and Bacilli numbers lower (P<0.01) after a 24 h wilt (Figures 2.2.2a - 2e). While the time to reach maximum temperature increased (P<0.01) with increasing DM concentration, wilt had little further effect on silage aerobic stability (Table 3.2.2).

Silage pH was lower (P<0.001) while lactic acid (P<0.01), acetic acid (P<0.001) and total fermentation product (P<0.01) concentrations were higher for the precision-chop silage in laboratory silos (PS), with values for the unchopped silage in laboratory silos (LS) and baled silage (BS) being similar (Table 3.2.1). The WSC concentration was lowest (P<0.001, Figure 3.2.3d) for the PS system followed by the LS and BS (70, 79 and 86 g/kg, respectively) systems, while NDFom content was highest (P<0.01) for the BS system with no difference observed between the other two ensiling systems (544, 536, 536 g/kg, respectively). Both Enterobacteria (P<0.001) and Clostridia (P<0.05) numbers were low in general but lowest in the PS (Figures 3.2.4b – 4c). BS was less stable on exposure to air at day 98 with a faster (P<0.001) time to onset of heating, a higher (P<0.01) maximum temperature rise recorded, and higher accumulated temperatures during both 120 and 192 h aerobiosis (Table 3.2.2).

Herbage pH and WSC concentration decreased (P<0.001), while silage buffering capacity, the concentration of fermentation products and ammonia-N concentration all increased (P<0.001), over the course of fermentation (Figures 3.2.1a – 1h; Table 3.2.3). The greatest increase (P<0.001) in lactic, acetic and propionic acids, and ethanol occurred between ensiling (day 0) and day 2. Butyric acid concentration was low over the course of the fermentation with the concentration only increasing (P<0.001) slightly toward the end of the ensilage period. The DMD and NDFom decreased (P<0.001), while ADFom (P<0.001) and ash (P<0.05) content increased during ensilage (Table 3.2.3). Lactic acid bacteria numbers increased (P<0.001) dramatically after ensiling with numbers at their highest after 14 days ensiling, and numbers then decreasing to day 98 (Figures 3.2.2a - 2e). Enterobacteria (P<0.001) and yeast (P<0.001) numbers peaked at day 2 of ensiling and then declined, but with yeast numbers increasing again slightly toward the end of the storage period. A similar extent of increase (P<0.001) in Clostridia numbers was observed from day 6 onward. Bacilli numbers declined (P<0.001) after ensiling and remained static throughout the remainder of the sampling period.

Silage pH was lower (P<0.05) for PS at each stage of wilting (Table 3.2.1). Lactic acid (P<0.001), acetic acid (P<0.01) and the total concentration of fermentation products were lowest (P<0.001) for the BS system in the unwilted and 48 h wilt treatments. In the 24 h wilted herbage, lactic acid concentration was highest in BS, while acetic acid concentration was similar for all systems. The proportion of lactic acid in fermentation products was lower (P<0.01) for the BS system after a 48 h wilt.

Clostridia numbers were generally low (< 2.0 cfu/g silage), with numbers being slightly higher (P<0.05) for BS after a 0 h wilt (Table 3.2.1). For the wilted silages, the BS system resulted in a higher (P<0.05) maximum temperature rise following exposure to air, while in the unwilted herbage the maximum temperature was similar for the BS and PS systems (Table 3.2.2). There were no further significant interactions (P>0.05) between these factors on any of the other variables measured.

Effluent was produced from the unwilted herbage only. The quantity of effluent produced was greater for the PS compared to LS system, with mean (s.d.) values on days 2, 6, 14, 35 and 98 of 0, 0, 7 (12.7), 18 (15.3) and 37 (29.6) g/kg herbage ensiled for the PS, and values of 0, 0, 0, 0 and 4 (7.4) g/kg for the LS, respectively. Effluent was not quantified from BS.

As wilting increased the herbage DM concentration, the extent and rate of decline in pH and WSC concentration decreased over time (P<0.001; Figure 3.2.1a, Table 3.2.3). The increase in buffering capacity and the concentration of fermentation products was greater (P<0.001) over time in the unwilted herbage (Figures 3.2.1b – 1h). With each 24 h increase in the duration of wilting, fermentation became more restricted. An increase (P<0.001) in ethanol and ammonia-N concentration was observed for the wilted herbages from day 35 to day 98, with the concentrations being similar at day 98 in the 24 h

wilted and unwilted silages. A slight increase ($P<0.001$) in butyric acid concentration was observed in the unwilted and 24 h wilted herbages after 14 days ensiling.
The DMD decreased ($P<0.01$) while ADFom ($P<0.001$) increased during ensiling, with the greatest changes occurring in the unwilted herbage. There was little change in NDFom concentration in the unwilted herbage during ensilage, while a decrease ($P<0.001$) was observed in the wilted herbages. Ash content increased ($P<0.001$) in the unwilted herbage during ensiling while little difference was observed for the wilted herbages. The CP content increased ($P<0.05$) in the unwilted herbage during ensilage, with a corresponding decrease in the 48 h wilted herbage. There was no significant interaction ($P>0.05$) between these factors for fresh weight loss.
The rate of increase ($P<0.001$) in lactic acid bacteria was similar for the unwilted and 24 h wilted herbage (Figures 3.2.2a - 2e). Although lactic acid bacterial numbers were greatest in the 48 h wilted herbage prior to ensiling, numbers were lowest in this treatment at each stage of ensiling. In the unwilted and 24 h wilted herbages, an increase ($P<0.001$) in Enterobacteria and yeast numbers was observed to day 2, followed by a rapid decline. The rate of decline in Enterobacteria numbers was highest for the unwilted herbage. Following the initial decline, yeast numbers increased after day 6 in all silages up to day 35, before continuing to increase up to day 98 in the 48 h wilt treatment only. After ensiling, Clostridia numbers increased ($P<0.01$) slightly in the unwilted and 24 h wilted herbage with a greater increase occurring for the 24 h wilt treatment. Bacilli numbers decreased ($P<0.001$) after ensiling, remaining static over the remainder of the storage period.

Herbage pH decreased faster ($P<0.05$) in PS, especially from day 0 to day 2, while the decrease was similar between the two other ensiling systems (Figure 3.2.3a). After day 2 for the BS system, little change in acetic acid concentration occurred, while the concentration of this acid continued to increase ($P<0.001$) in the LS and PS systems over time (Figures 3.2.3b – 3d). In the LS system the initial increase ($P<0.001$) in ethanol concentration to day 14 was slower than in other systems, but the concentration continued to increase up to day 98. The rate of decline in WSC was faster ($P<0.05$) for the PS system especially in the early days of ensiling. However, the final WSC concentration on day 98 was similar for LS and PS, but with a higher concentration remaining in the BS system. The NDFom content decreased ($P<0.01$) during ensiling by a similar concentration for the BS and PS systems, while the decrease in the LS silage was lower (26 - 27 compared to 10 g/kg DM, data not shown). There were no further significant interactions ($P>0.05$) between these factors on any of the other chemical variables measured.
Lactic acid bacteria numbers increased ($P<0.05$) faster in the PS system up to day 6, with numbers peaking in all systems after 14 days ensiling, and numbers for all systems being similar on day 98 (Figures 3.2.4a - 4d). The decrease ($P<0.001$) in *Enterobacteria* numbers over time was greatest for the PS with numbers detected on day 98 in baled silage only. Yeast numbers increased ($P<0.01$) slightly in all systems towards the end of ensiling but with the greatest increase occurring in the BS system. After day 6 of ensiling for the BS and LS systems Clostridia numbers increased ($P<0.01$) slightly, to values just greater than 2.0 cfu/g silage at the end of the sampling period.
Acetic acid concentration increased ($P<0.001$) rapidly in the unwilted herbage, but with a slower increase observed for the wilted treatments in the early stages of ensilage (Figure 3.2.5a). After 98 days ensilage, the final acetic acid concentration was lower with increasing duration of wilt (Figure 3.2.5b). An exception to this was in the unwilted BS system where the concentration was similar to that of the 24 h wilted silage after a decrease from day 35. These trends were reflected in similar changes ($P<0.001$) in total fermentation products.
Lactic acid bacteria numbers in the unwilted LS and PS systems peaked ($P<0.01$) on day 6 of ensiling, while in the BS system numbers peaked on day 14 (Figure 3.2.5c). For the 24 h wilt treatment, lactic acid bacteria numbers for all systems also peaked on day 14, but with numbers for the BS system being lower. At the end of the ensiling period, lactic acid bacteria numbers were highest in the unwilted silage, with numbers for the wilted silages being similar. There were no further significant interactions ($P>0.05$) on any of the other variables measured.
Conclusions: The onset of fermentation and the decline in pH were slower in baled compared to precision-chop silages. This permits the continuation of plant enzyme activity (especially in wetter herbages) and the persistence of greater numbers of Enterobacteria in the early stages of ensilage, both of which could impede achieving a successful preservation. The latter effect is further accentuated in baled silage, where wilting is an integral part of production, by the restrictive impact of wilting on fermentation. Furthermore, these effects and the numbers of undesirable microorganisms would be greater for farm silages where storage conditions can sometimes be less than ideal. The combined effects of factors such as the extent of wilting, harvester type (e.g. differ in the extent of physical disruption caused to herbage) and the effectiveness of excluding oxygen combine to create conditions within bales that are less conducive to inhibiting the activity of undesirable microorganisms than occurs in precision-chop silage. Thus, technologies are needed to improve the fermentation in baled silage. In general, the use of laboratory silos for the LS treatment proved to be a relatively useful model for BS

when contrasting with PS.

Table 3.2.1: Significant interactions between wilt and ensiling system – differences in silage chemical, fermentation and microbial composition (g/kg DM, except pH, unless otherwise stated)

Wilt (h)	Ensiling system	pH	Lactic acid	Acetic acid	FP	L/FP (g/g)	CP	Clostridia (log_{10} cfu/g silage)
0	BS	4.42	69	14.6	98	0.70	144	1.9
0	LS	4.40	77	18.1	111	0.68	152	1.3
0	PS	4.29	82	19.3	115	0.71	147	1.2
24	BS	4.94	47	9.1	69	0.63	147	1.8
24	LS	5.09	37	9.2	57	0.60	141	2.0
24	PS	4.84	44	9.0	64	0.63	145	1.6
48	BS	5.18	14	5.5	27	0.47	148	1.4
48	LS	5.07	21	6.7	34	0.57	149	1.3
48	PS	4.92	22	6.8	35	0.56	148	1.4
	s.e.m.[#]	0.040	2.0	0.60	2.3	0.016	2.1	0.13
Levels of significance								
Wilt		***	***	***	***	***	*	***
Ensiling system		***	**	***	**	*	NS	*
Wilt x ensiling system		*	***	**	***	***	*	*

BS = baled silage, LS = long grass in laboratory silos, PC = precision-chop grass in laboratory silos, FP = total fermentation products, L/FP = proportion of lactic acid in fermentation products, CP = crude protein, * = P < 0.05, ** = P < 0.01, *** = P < 0.001, [#] s.e.m. relates to the interaction between wilt and ensiling system

Table 3.2.2: Silage aerobic stability data (day 98 only) for 0 h, 24 h and 48 h wilted silages ensiled in bales, as long grass in laboratory silos and as precision chopped grass in laboratory silos

Wilt (h)	Ensiling system	Time to temp. rise > 2°C (h)	Maximum temperature rise (°C)	Time to maximum temperature (h)	Accumulated temp. rise to 120 h (°C)	Accumulated temp. rise to 192 h (°C)
0	BS	67	13	103	28	50
0	LS	131	8	192	4	17
0	PS	105	14	137	12	29
24	BS	57	21	185	18	65
24	LS	192	1	192	2	4
24	PS	103	12	171	9	35
48	BS	60	15	189	17	43
48	LS	142	10	192	2	17
48	PS	153	5	192	4	12
	s.e.m.[#]	23.5	2.8	16.7	5.8	8.9
Levels of significance						
Wilt		NS	NS	**	NS	NS
Ensiling system		***	**	NS	**	***
Wilt x ensiling system		NS	*	NS	NS	NS

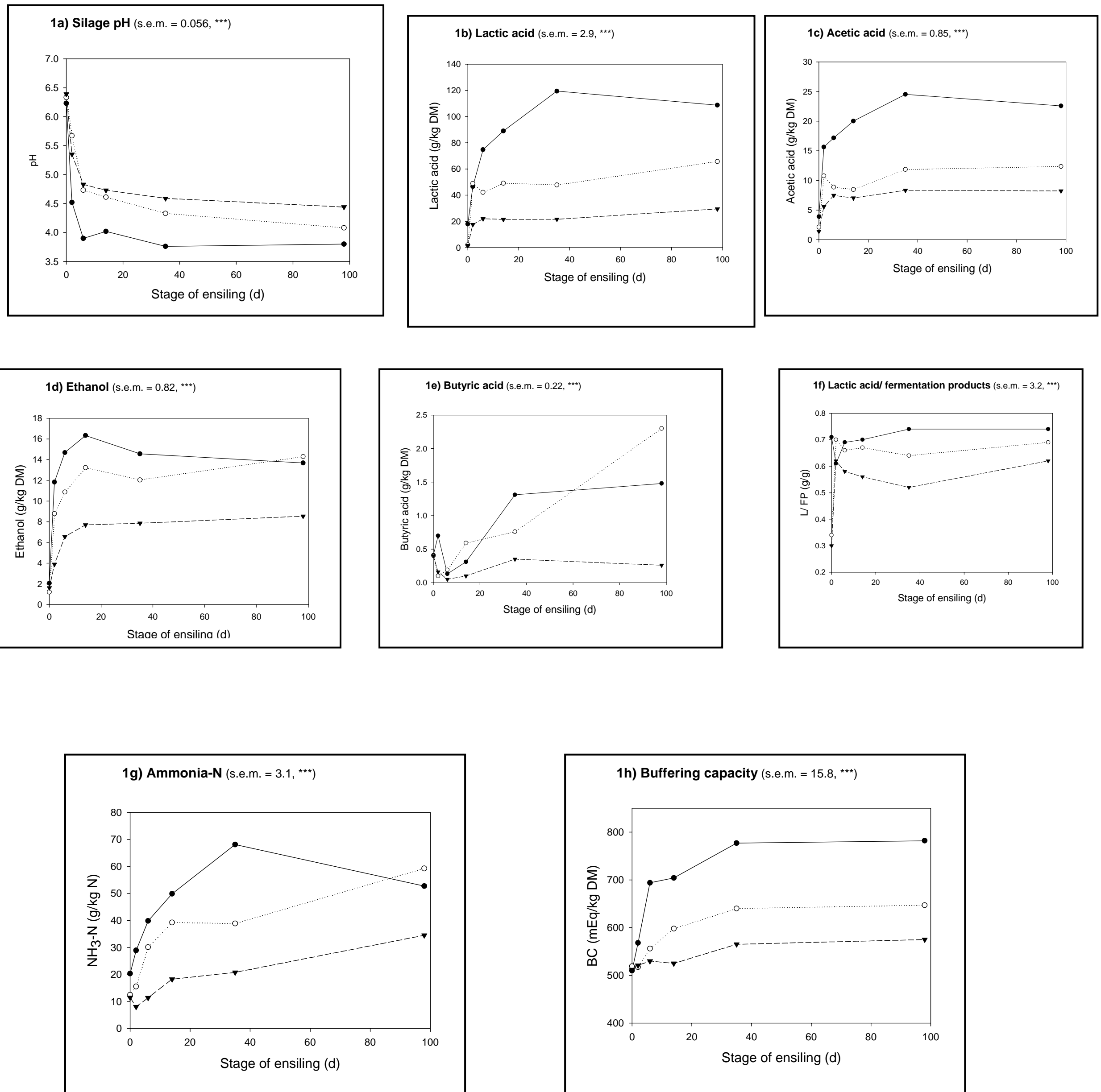
BS = baled silage, LS = long grass in laboratory silos, PC = precision-chop grass in laboratory silos, * = P < 0.05, ** = P < 0.01, *** = P < 0.001, [#] s.e.m. relates to the interaction between wilt and ensiling system

Table 3.2.3: Interactions between wilt and stage of ensiling – changes in silage chemical composition (g/kg DM, unless otherwise stated) and fresh weight loss over the course of fermentation in 0 h, 24 h wilted and 48 h wilted silages.

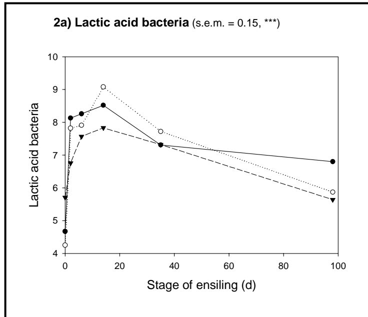
		DM (g/kg)	WSC	DMD[$] (g/kg)	NDFom[$]	ADFom[$]	Ash[$]	CP	Fresh weight loss[£] (g/kg)
Wilt (h)	Stage of ensiling (d)								
0	0	185	202	818	530	278	89	138	-
0	2	172	102	-	-	-	-	151	5
0	6	169	61	-	-	-	-	152	6
0	14	177	43	-	-	-	-	150	8
0	35	177	25	-	-	-	-	148	9
0	98	168	6	781	527	327	104	147	13
24	0	303	178	805	554	291	94	139	-
24	2	309	121	-	-	-	-	150	3
24	6	293	70	-	-	-	-	148	7
24	14	296	37	-	-	-	-	144	11
24	35	288	20	-	-	-	-	148	11
24	98	291	18	803	519	302	97	138	12
48	0	406	151	804	564	301	97	152	-
48	2	436	132	-	-	-	-	157	5
48	6	410	90	-	-	-	-	154	10
48	14	415	72	-	-	-	-	144	11
48	35	415	46	-	-	-	-	145	10
48	98	415	32	790	538	313	93	139	16
	s.e.m.[#]	8.2	4.1	4.4	2.9	3.5	2.3	3.0	2.2
Levels of significance									
Wilt		***	***	NS	***	*	NS	*	NS
Stage of ensiling		NS	***	***	***	***	*	***	NS
Wilt x stage of ensiling		NS	***	**	***	***	***	*	NS

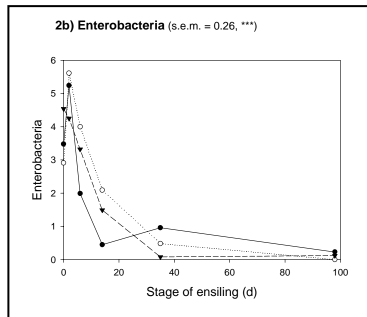
DM = dry matter, WSC = water soluble carbohydrate, DMD = dry matter digestibility, NDFom = neutral detergent fibre, ADFom = acid detergent fibre, CP = crude protein, fresh weight loss, [$] = variables measured on day 0 and day 98 only, [£] = variable not measured on day 0, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, NS = not significant, [#] = s.e.m. relates to the interaction between wilt and stage of ensiling

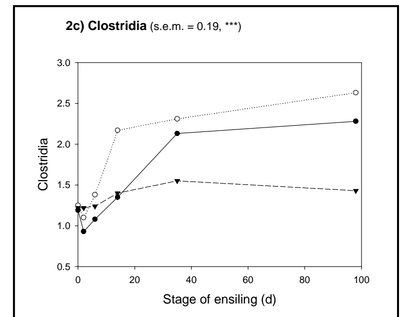
Figures 3.2.1a – 1i: Significant interactions between wilt and stage of ensiling – changes in silage fermentation variables, standard error of the mean (s.e.m.) and significance levels (*** = $P < 0.001$), over the course of fermentation in 0 h (-●-), 24 h (-○-) and 48 h (-▼-) wilted silages.

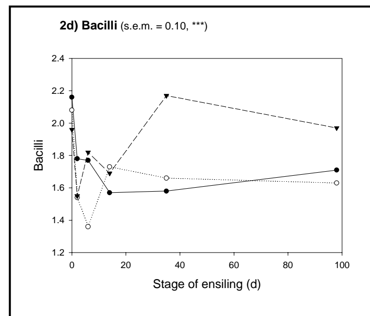


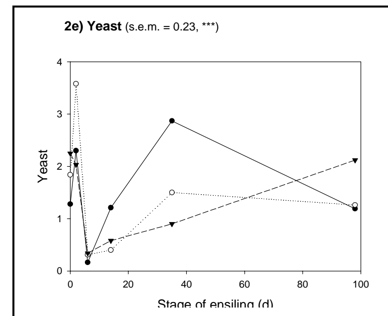
Figures 3.2.2a – 2e: Significant interactions between wilt and stage of ensiling – changes in silage microbial composition ($\log_{10}$ cfu/g silage), standard error of the mean (s.e.m.) and significance levels (*** = P < 0.001), over the course of fermentation in 0 h (-●-), 24 h (-○-) and 48 h (-▼-) wilted silages.



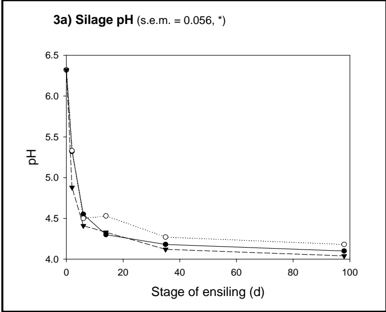


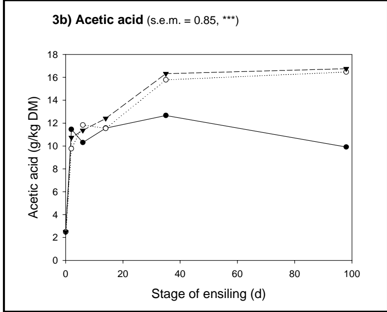


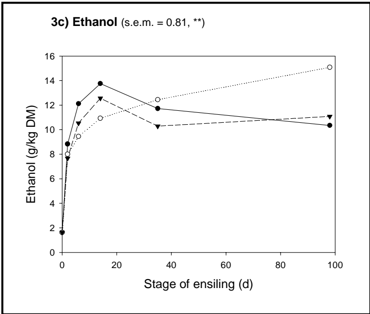


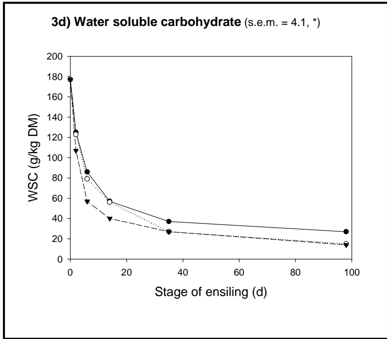


Figures 3.2.3a – 3d: Significant interactions between ensiling system and stage of ensiling – changes in silage fermentation variables and water soluble carbohydrate concentration, standard error of the mean (s.e.m.) and significance levels (* = P < 0.5, ** = P < 0.01, *** = P < 0.001), over the course of fermentation in baled (-●-), long (-○-) and precision-chop (-▼-) silages

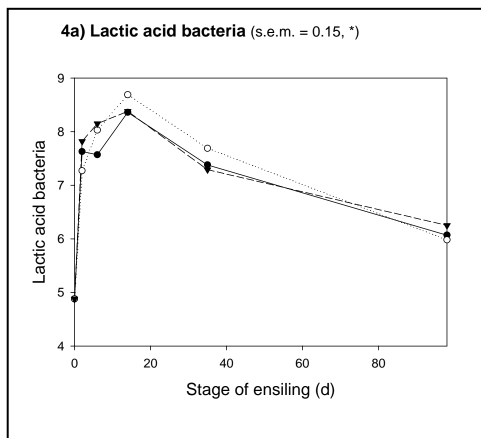


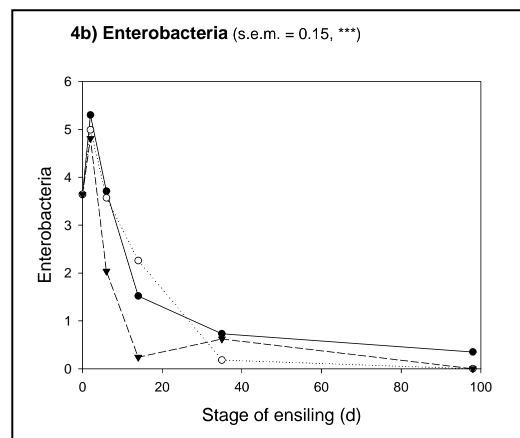


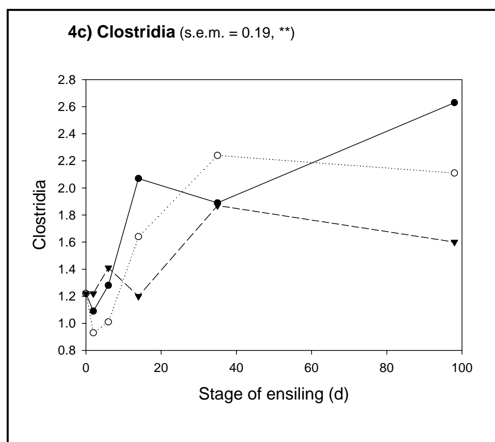


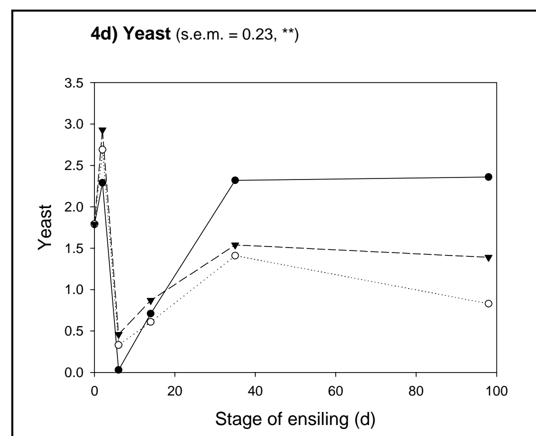


Figures 3.2.4a – 4d: Significant interactions between ensiling system and stage of ensiling – changes in silage microbial composition (log_{10} cfu/g silage), standard error of the mean (s.e.m.) and significance levels (* = P < 0.5, ** = P < 0.01, *** = P < 0.001), over the course of fermentation in baled (-●-), long (-○-) and precision-chop (-▼-) silages









Experiment 3.3. The relative impacts of wilting, chopping, compaction and air infiltration on the conservation characteristics of ensiled grass

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The aim of this study was to elucidate the relative contributions of wilting, chopping, compaction and air infiltration to the conservation characteristics of ensiled grass. This information, obtained under controlled laboratory conditions, should contribute to understanding why the conservation characteristics of individually wrapped bales of unchopped silage often differ from those of other more conventional silages.

Materials and methods: The experiment was organised in a 3 (duration of wilt treatments) x 2 (chop treatments) x 2 (compaction treatments) x 2 (air infiltration treatments) factorial arrangement of treatments. Grass was wilted for 0, 24 or 48 h, was harvested either unchopped or chopped, was ensiled with or without compaction, and was stored with or without air infiltration. Twenty-four laboratory silos were filled on each of three separate days corresponding to three different wilting periods (i.e. DM concentrations). Microbiological and biochemical profiles were obtained after 100 days ensilage, and the subsequent aerobic stability of the silages was assessed. .

An homogenous plot of *Lolium perenne* (cv. Fennema) was mown (Pottinger, Nova 310T conditioner mower) on the 27 July 2004 (9 week regrowth). There was no rainfall during wilting and harvesting, and drying conditions were generally excellent. .

Six representative grass grab samples were taken after 0, 24 and 48 h field wilting to characterise the grass at time 0. Subsamples of this herbage were used to determine herbage DM concentration by microwave drying prior to silo filling. .

At the same time, twenty four representative grab samples (*ca*. 7 kg each) were taken after each period of wilting and transported to the silage research laboratory for silo filling. A constant weight of 0.8 kg herbage DM was ensiled in each laboratory silo. On each ensiling day, the sampled herbage was used to fill 24 laboratory pipe silos (height = 0.75 m, internal diameter = 0.152 m, internal volume = 13.6 l) with the herbage for twelve of these silos being precision-chopped (Pottinger, Mex VI) immediately prior to ensiling. The chopping knife number and feed roller speeds were chosen, according to the manufacturers instructions, to give a theoretical chop length of 19 mm. The remaining twelve silos were filled with unchopped herbage. The silos were filled and manually compressed using a repeatable procedure to a constant depth. Compaction was achieved in half of the silos by the inclusion of a 21 kg steel weight (diameter = 0.1 m) directly on the herbage to exert continual vertical pressure, while the remainder had no weight included. The weight was fully carried by the silage with a pressure of 11.66 kPa. In the uncompacted silos, the remainder of the silo was void or empty space. Air infiltration was achieved in half of the silos by incomplete sealing, with the screw-on base and lid of these silos only being hand tightened. Complete sealing was achieved by mechanically tightening the base and lid of the remainder of the silos. The use of rubber O-rings in the screw-on base and lid of all silos ensured complete sealing when mechanically tightened. Each silo lid was fitted with a water filled gas release valve. Each treatment combination was replicated three times, with the total of 72 laboratory silos being stored at 15 ºC for 100 days.

Effluent (if any) was collected and weighed at silo opening and the final settled depth and weight of the silage were recorded. After thorough aseptic mixing, one sample per silo was taken and all samples were stored at 4 °C prior to microbiological analyses and at –18 °C prior to chemical analyses.

Once each silage was sampled, the remainder was assessed for aerobic stability and deterioration. The volume of the silo occupied by the silage was calculated using the measurement of the final settled depth of silage in the silo. By assuming that the density of herbage DM = 1.5 kg l^{-1} and of water = 1 kg l^{-1}, the volume of the silage matter (i.e. DM plus water, excluding void spaces) was obtained. From the latter two values the silage void or pore space was calculated.

Results: Herbage DM concentrations increased as the duration of wilting was extended, with values of 185, 465 and 652 (s.e.m. = 2.9, $P<0.001$). The pH was numerically higher with wilted than unwilted herbage (Table 3.3.1). Wilting was rapid, with mean hourly rates of increase in herbage DM concentration of 11.7 and 7.8 g/kg during the first and second 24 h wilting periods, respectively reflecting excellent drying conditions. Thus, the a_w of the herbage at the three stages of wilting can be calculated as approximately 0.993, 0.974 and 0.944, respectively.

The buffering capacity of the herbage was higher in the wilted material, while the WSC concentration was lower after each stage of wilting. Herbage DMD was lower and ash concentration tended to be higher as the duration of wilting was extended, while wilting had little effect on fibre indices. The numbers of lactic acid bacteria, yeast and *Enterobacteria* on herbage prior to ensiling were numerically highest after the 24 h wilting period and lowest after the 48 h wilt. In contrast, with extensive wilting (48 h) the numbers of both spores of Clostridia and Bacilli were slightly higher.

Wilt: Silage DM and pH were highest (P<0.001) after a 48 h wilt (Tables *3.3.*2 and *3.3.*3). The unwilted herbage underwent the most extensive fermentation, with the lowest (P<0.001) silage pH and highest (P<0.001) concentration of total fermentation products (lactic acid, acetic acid, propionic acid, butyric acid and ethanol). Lactic acid was the dominant (P<0.001) fermentation product in the unwilted and 24 h wilted silages while after an extensive wilt only low concentrations of lactic acid were detected making up only proportionally 0.38 of the fermentation products. Acetic, propionic and butyric acid concentrations together with silage buffering capacity decreased (P<0.001), while the final WSC concentration increased (P<0.001), with increasing DM concentration. Ethanol (P<0.05) and ammonia-N (P<0.001) concentration were similar in the unwilted and 24 h wilted silages with a decrease observed after a 48 h wilt. The NDF concentration increased (P<0.001) in the wilted silages. Wilting had a significant effect on all the microorganisms measured (Table *3.3.*4). Lactic acid bacteria, yeast, Clostridia and Bacilli numbers were higher in silage than on herbage prior to ensiling (Table *3.3.*1), while Enterobacteria numbers were lower. As with the effect of wilting on fresh grass, the highest numbers of lactic acid bacteria (P<0.001) and Enterobacteria (P<0.01) in silage were observed with the 24 h wilt treatment. Yeast numbers on silage increased (P<0.001) with increasing DM concentration; with the highest numbers recorded in the 48 h wilt treatment. Clostridia numbers were highest (P<0.001) in unwilted silage and a decrease was observed due to wilting, while in contrast Bacilli numbers were lowest (P<0.001) in the unwilted silage. Effluent was produced only in the unwilted silage with a value of 39 g/kg grass ensiled. Silage pore space increased (P<0.001) with increasing DM concentration, the largest increase being with the first 24 h wilt (Table *3.3.*5). On exposure to air at silo opening, the unwilted silage was less stable compared to the wilted silages, with a lower (P<0.001) time to onset of heating and a higher (P<0.001) temperature rise.
Chop: Although silage DM concentration was higher (P<0.05) and pH lower (P<0.05) in the precision-chop silage, chopping had little effect on silage variables measured (Tables 3.3.2 – 3.3.4). However, ethanol (P<0.01), butyric acid (P<0.05) and ammonia-N (P<0.01) concentrations were all slightly higher in the unchopped material. The final settled height of the silage in the silo and its pore space were significantly lower (P<0.001) for the precision-chop treatment (Table 3.3.5). However the magnitude of this effect was modest.Compaction: Although silage pH was lower (P<0.05) and DM concentration higher (P<0.01) for the compacted material, the presence of compaction had no effect (P>0.05) on any of the fermentation products measured (Table 3.3.3). Compaction with a weight caused an increase in silage NDF (P<0.01) and ADF (P<0.05) concentrations, while reducing (P<0.05) the ash concentration (Table 3.3.2). The only microorganisms influenced by compaction were Bacilli, which were higher (P<0.01) in the uncompacted silage (Table 3.3.4). The inclusion of a weight to compact the herbage resulted in an increase in effluent production (21 versus 5 g/kg grass ensiled), a reduction (P<0.001) in the height of the silage in the silo and a decrease (P<0.001) in silage pore space (Table 3.3.5).
Air infiltration: For the non-mechanically sealed silos, a small amount of mould growth was visible at the top and sometimes at the bottom of the silage mass at silo opening, indicating that air infiltrated the silos during storage. The ingress of air into the silos caused a decrease (P<0.001) in silage DM concentration and an increase (P<0.001) in pH (Tables 3.3.2 and 3.3.3). Lactic acid and WSC concentrations, and silage buffering capacity were lower (P<0.001) when air infiltrated, while butyric acid and ammonia-N concentrations were both higher (P<0.001). Air infiltration did not alter (P>0.05) ethanol, acetic or propionic acid concentrations. Silage DMD was lower (P<0.001) when air was allowed to infiltrate the silos, while NDF, ADF and ash concentrations were higher (P<0.001). Air increased (P<0.001) the numbers of all five microorganisms enumerated (Table 3.3.4) and decreased (P<0.001) the final settled height of the silage (Table 3.3.5). The silages subjected to the air infiltration treatment proved slightly less stable after silo opening, with a faster (P<0.01) rise in temperature. However, the increase in accumulated temperature rise to 120 h, although statistically significant (P<0.05), was still relatively small.
Wilt x chop: In the unwilted silage, butyric acid concentration and Clostridia numbers were higher (P<0.05) for the unchopped compared to the precision-chopped treatments. This trend was not evident in the wilted silages, with wilting resulting in a decrease in both butyric acid concentration and Clostridia numbers, and values for both chop treatments being similar. Chopping herbage prior to ensiling resulted in a reduced (P<0.05) buffering capacity with the unwilted silage, but not with silages made from wilted herbages. The ADF concentration was reduced (P<0.05) in chopped silages made after a 0 and 24 h wilt, but not for the 48 h wilted herbage. Effluent production in the unwilted material was greater for the precision-chop (56 g/kg grass ensiled) compared to the unchopped (23 g/kg) herbage. In the unwilted treatment, chopping resulted in a more compact silage with a lower (P<0.001) percentage pore space, while these trends were nullified by wilting.
Wilt x compaction: Compaction for the unwilted and 48 h wilt treatments resulted in higher (P<0.05) DM concentrations. The presence of compaction also resulted in a higher (P<0.05) final WSC concentration in the 48 h wilted silage. Lactic acid bacterial numbers were higher (P<0.01) when

unwilted silage was compacted while a decrease was observed with compaction of the 48 h wilted herbage. *Clostridia* numbers declined ($P<0.05$) in response to compaction with the 0 and 24 h wilted silages, but not with the 48 h wilted silages. Compaction resulted in a decrease ($P<0.05$) in ash concentration in the unwilted silage, with the opposite observed for the 48 h wilt treatment. Effluent production from the unwilted herbage was higher in the presence of compaction with a weight (63 versus 15 g/kg grass ensiled). The effect of the inclusion of a weight on decreasing silage pore space decreased ($P<0.001$) with wilting. In the 0 h wilt treatment silage fresh weight loss was higher ($P<0.001$) with the compacted herbage, but this trend was reversed for the 48 h wilt treatment where the loss was greater in the absence of compaction.

Wilt x air infiltration: Silage pH increased ($P<0.001$) with increasing DM concentration, but with the pH being higher in the unsealed compared to the completely sealed silos at each DM concentration. Lactic acid and WSC concentrations were lower ($P<0.001$), while ammonia-N ($P<0.001$) and ash ($P<0.05$) concentrations were higher in the unsealed silos at each DM concentration, although the magnitude of this effect varied with the extent of wilting. Ethanol concentration was lower ($P<0.001$) in the presence of air in both wilted silages, while an increase was observed in the unwilted silage. Butyric acid concentration was highest ($P<0.001$) in the unwilted silage, with the value more than doubling due to the presence of air. In contrast, air infiltration had no effect on butyric acid concentration in the wilted silages. For both the unwilted and 24 h wilted silages, buffering capacity decreased ($P<0.001$) in the presence of air while in the 48 wilted material the opposite occurred. While lactic acid bacterial numbers were similar in the presence and absence of air infiltration for the unwilted silage, numbers increased ($P<0.001$) in the wilted silages in response to air infiltration. The extent of this rise became greater as silage DM concentration increased. Bacilli ($P<0.01$) numbers increased the most in response to air infiltration with the 24 h wilted treatment. Clostridia numbers were also higher ($P<0.001$) with air infiltration, but the magnitude of increase declined with increasing silage DM concentration. Yeast numbers increased ($P<0.001$) in the wilted silages in the presence of air but not in the unwilted silage, and were highest after a 48 h wilt. While the silage fresh weight loss was increased by air infiltration at all DM concentrations, the level of increased loss was greatest ($P<0.001$) with the 48 h wilt treatment. For the unwilted silages, the silages subjected to air infiltration were slightly more stable after silo opening, with a slower ($P<0.001$) duration to a temperature rise. The opposite was the case for the 24 h wilt treatment.

Chop x compaction: In the absence of compaction, lactic acid bacterial numbers were higher ($P<0.05$) in the unchopped silage compared to the chopped silage. When compacted, the settled height ($P<0.001$) and pore space ($P<0.01$) were both reduced less by chopping than when the silage was not compacted.

Chop x air infiltration: Air infiltration resulted in an increase in ammonia-N concentration for both chop treatments but with the increase being greater ($P<0.05$) in the unchopped herbage. In the absence of air infiltration, lactic acid bacterial numbers were slightly higher ($P<0.01$) in the unchopped compared to the precision-chopped herbage, with the opposite occurring when air infiltrated the silo.

Compaction x air infiltration: Silage pH was higher in the presence of air infiltration and this increase in pH was more pronounced ($P<0.05$) in the absence of compaction. Silage DM concentration and buffering capacity decreased with air infiltration, with the largest decrease ($P<0.05$) occurring in the absence of compaction. Acetic acid concentration was higher ($P<0.01$) in the sealed silos with no compaction and in the unsealed silos in the presence of compaction. NDF concentration increased ($P<0.05$) with air infiltration, especially in the presence of compaction. Numbers of lactic acid bacteria ($P<0.05$), *Clostridia* ($P<0.05$) and *Bacilli* ($P<0.001$) increased in the unsealed silos, with the greatest increases occurring in the absence of compaction. Air ingress during ensilage had a more detrimental effect on the post-ensilage aerobic stability of the uncompacted rather than compacted silage.

Wilt x chop x compaction: Within the 24 h wilted herbage, the DM concentration of the unchopped, compacted silage was lower ($P<0.01$) than for other treatments, while in the 48 h wilt treatment the unchopped uncompacted herbage produced silage with a lower DM concentration. The ash concentration for the unwilted silage was higher ($P<0.05$) for the uncompacted herbage regardless of chop treatment. A similar trend was evident for the 48 h wilted, unchopped silage, while this did not occur with the 24 h wilted silage. *Bacilli* numbers were lowest ($P<0.01$) for the unwilted and 24 h wilted precision-chop silages subjected to compaction. In the 48 h wilted silages, compaction also caused a decrease in *Bacilli* numbers, but with the lowest numbers present in the silage made from unchopped herbage. The effect of chopping in reducing ($P<0.05$) the final settled height of the silage was only evident in the unwilted material and was nullified by the inclusion of a weight to compress the herbage. There was no significant difference in silage pore space.

Wilt x chop x air infiltration: No significant effect was recorded for any of the variables measured.

Wilt x compaction x air infiltration: In the 24 h wilted treatment silage DM concentration was lower ($P<0.05$) with air infiltration regardless of the compaction treatment, while in the 48 h wilt treatment the decrease in silage DM was more pronounced in the absence of compaction. In the wilted herbages,

regardless of the presence or absence of compaction, the ethanol concentration was lower ($P<0.05$) with air infiltration. However, in the unwilted herbages, air infiltration had the opposite effect with the increase in ethanol concentration being particularly evident in the presence of compaction. Acetic acid concentration was highest ($P<0.05$) for the unwilted silage, in the uncompacted sealed silos in particular, while there was no difference observed in the wilted silages. Silage height and pore space decreased ($P<0.001$) more in the sealed silos in the presence of compaction compared to the silos subjected to air infiltration. In the unwilted silages subjected to air infiltration, the addition of a weight to compact the herbage resulted in a more stable silage post-ensiling with a lower ($P<0.05$) accumulated temperature rise to 120 h. Silage fresh weight loss was greater ($P<0.001$) in the unwilted herbage with air infiltration, especially in the presence of compaction while in the 48 h wilted silage the loss was greater in the absence of compaction.
Chop x compaction x air infiltration: The NDF and ADF concentrations were higher ($P<0.05$) with air infiltration in the presence and absence of compaction but with the values being slightly higher with compaction.

Wilt x chop x compaction x air infiltration: In the wilted silages in particular, lactic acid bacterial numbers were higher ($P<0.01$) with air infiltration and in the absence of compaction. Chopping had little effect, while numbers were lowest in the 48 h wilted silage stored without air infiltration. As herbage DM concentration increased due to wilting a larger decrease ($P<0.05$) in WSC concentration with air infiltration occurred.

Conclusions: The present experiment indicates that the range of herbage DM concentrations and the impact of low and high air ingress had a much greater effect on silage conservation characteristics than the compaction or chopping treatments studied. The main interactions were between the extents of wilting and air infiltration, wilting and compaction, and compaction and air infiltration. Air infiltration, as allowed in this trial, had a greater effect on in-silo losses than any of the other factors studied.
A surprising outcome with many of the conservation variables studied was the absence of significant interactions between most of the factors. In many cases this appeared to be related to the absence of pore space effects perhaps limiting the extent of air infiltration through the silage. Pore space was not greatly affected by either chopping or compaction and consequently these factors would have had little effect on air ingress.
The overwhelming objective with the baled silage system in particular, must therefore be to rapidly achieve adequately anaerobic conditions and maintain them thereafter. Failure to achieve this will lead to progressively greater losses, especially with drier herbage. The impacts of forage chopping or compaction are relatively minor if anaerobic conditions prevail. However, the evidence from Irish farm silages is that the technologies employed to achieve and maintain anaerobic conditions may be less successful with baled compared to conventional silages.

Table 3.3.1: The chemical (g/kg DM, unless otherwise stated) and microbiological composition (log_{10} cfu/g) of grasses prior to ensiling (s.d. in parenthesis)

	Duration of wilt (h)		
	0	24	48
pH	6.13 (0.050)	6.48 (0.080)	6.37 (0.175)
Dry matter (g/kg)	185 (4.7)	465 (7.7)	652 (8.2)
Chemical composition			
Buffering capacity (m. Eq/kg DM)	367 (7.1)	438 (8.9)	428 (17.9)
Water soluble carbohydrate	171 (9.4)	147 (7.5)	133 (5.4)
Dry matter digestibility (g/g)	0.775 (0.0032)	0.757 (0.0085)	0.748 (0.0129)
Neutral detergent fibre	529 (6.1)	526 (6.7)	532 (9.5)
Acid detergent fibre	291 (3.1)	292 (3.3)	299 (10.2)
Ash	89 (1.4)	92 (2.6)	97 (5.7)
Crude protein	135 (3.6)	145 (5.8)	150 (3.2)
Lactic acid bacteria	4.2 (0.09)	5.4 (0.13)	4.1 (0.39)
	4.1 (0.19)	4.7 (0.21)	3.6 (0.60)
	1.6 (0.15)	1.7 (0.26)	2.2 (0.25)
Bacilli	2.6 (0.58)	2.5 (0.21)	2.8 (0.34)
Yeast	2.5 (0.23)	3.3 (0.29)	2.0 (0.55)

Table 3.3.2: Treatment effects on some silage chemical composition indices (g/kg DM, unless otherwise stated)

Treatment				DM	DMD	NDF	ADF	Ash	CP	WSC
Wilt (h)	Chop	***Compaction***	Air							
0	UC	NW	NA	179	0.753	519	322	96	149	17
0	PC	NW	NA	173	0.753	496	302	101	144	16
0	UC	NW	AI	151	0.694	516	323	114	173	16
0	PC	NW	AI	156	0.694	523	324	114	164	14
0	UC	W	NA	179	0.743	523	323	94	147	16
0	PC	W	NA	182	0.745	521	317	96	146	15
0	UC	W	AI	179	0.718	547	339	97	153	16
0	PC	W	AI	183	0.741	534	324	99	147	13
24	UC	NW	NA	451	0.753	550	319	94	149	37
24	PC	NW	NA	471	0.755	539	314	94	147	37
24	UC	NW	AI	413	0.708	551	321	109	165	27
24	PC	NW	AI	401	0.678	553	323	113	164	26
24	UC	W	NA	452	0.742	547	316	101	150	38
24	PC	W	NA	472	0.742	539	309	91	148	37
24	UC	W	AI	381	0.709	571	345	119	166	23
24	PC	W	AI	424	0.713	549	324	110	163	28
48	UC	NW	NA	646	0.721	547	312	94	145	92
48	PC	NW	NA	645	0.737	558	311	93	149	86
48	UC	NW	AI	512	0.682	559	322	127	178	32
48	PC	NW	AI	571	0.698	553	325	112	168	43
48	UC	W	NA	651	0.734	551	309	91	146	93
48	PC	W	NA	648	0.735	549	310	91	141	103
48	UC	W	AI	600	0.693	575	324	104	162	39
48	PC	W	AI	585	0.703	563	325	113	167	44
			s.e.m.$	13.8	0.0144	6.3	4.4	4.3	3.6	2.9
Levels of significance										
Wilt				***	NS	***	NS	NS	*	***
Chop				*	NS	NS	**	NS	*	NS
Compaction				**	NS	**	*	*	**	NS
Air infiltration				***	***	***	***	***	***	***
Wilt x chop				NS	NS	NS	*	NS	NS	NS
Wilt x compaction				*	NS	NS	NS	*	*	*
Wilt x air infiltration				***	NS	NS	NS	*	*	***
Chop x compaction				NS	NS	NS	NS	NS	NS	NS
Chop x air infiltration				NS	NS	NS	NS	NS	NS	NS
Compaction x air infiltration				*	NS	*	NS	NS	**	NS
Wilt x chop x compaction				**	NS	NS	NS	*	NS	NS
Wilt x chop x air infiltration				NS	NS	NS	NS	NS	NS	NS
Wilt x compaction x air infiltration				*	NS	NS	NS	NS	NS	NS
Chop x compaction x air infiltration				NS	NS	*	*	NS	NS	NS
Wilt x chop x compaction x air infiltration$				NS	NS	NS	NS	NS	NS	*

UC = unchopped, PC = precision-chopped; NW = no compression weight, W = compression weight; NA = no air infiltration, AI = air infiltration; DM = dry matter (g/kg), DMD = dry matter digestibility (g/g), NDF = neutral detergent fibre, ADF = acid detergent fibre, CP = crude protein, WSC = water soluble carbohydrates; NS = not significant, * = P < 0.05, ** = P < 0.01, *** = P < 0.001, $ s.e.m. relates to 4 factor interaction

Table 3.3.3: Treatment effects on silage pH, buffering capacity, ammonia-N and fermentation products (g/kg DM, unless otherwise stated)

Treatment				pH	LA	Eth	AA	PA	BA	L: FP	NH_3-N	BC
Wilt (h)	Chop	***Compaction***	Air									
0	UC	NW	NA	3.77	165	10.1	31	5.5	7.2	0.76	72	975
0	PC	NW	NA	3.80	162	1.9	33	4.8	1.5	0.80	60	934
0	UC	NW	AI	4.43	100	8.1	23	5.1	8.6	0.69	131	833
0	PC	NW	AI	4.27	96	5.5	18	4.1	7.3	0.79	79	788
0	UC	W	NA	3.67	160	5.4	26	4.3	4.6	0.80	64	982
0	PC	W	NA	3.77	149	3.9	25	2.8	2.7	0.82	59	907
0	UC	W	AI	3.87	127	19.6	29	6.0	14.2	0.65	97	870
0	PC	W	AI	3.80	123	11.9	27	4.6	9.4	0.70	62	875
24	UC	NW	NA	4.30	50	12.5	12	1.7	1.4	0.65	47	589
24	PC	NW	NA	4.20	57	7.7	13	1.8	1.4	0.71	44	604
24	UC	NW	AI	6.43	28	6.0	10	1.3	1.4	0.64	112	535
24	PC	NW	AI	5.93	44	4.0	9	1.4	1.5	0.74	117	550
24	UC	W	NA	4.30	46	10.7	7	0.6	0.7	0.71	44	586
24	PC	W	NA	4.17	62	10.2	10	1.0	0.9	0.74	46	601
24	UC	W	AI	6.57	37	3.9	7	1.3	1.7	0.74	143	552
24	PC	W	AI	5.47	41	4.3	9	0.6	1.0	0.74	90	578
48	UC	NW	NA	5.43	7	5.9	5	0.1	0.9	0.38	13	481
48	PC	NW	NA	5.23	8	10.0	6	0.4	0.9	0.31	16	474
48	UC	NW	AI	7.63	5	1.8	7	0.6	0.7	0.31	56	509
48	PC	NW	AI	6.50	7	1.8	7	1.0	0.8	0.39	32	507
48	UC	W	NA	5.40	7	7.2	3	0.1	0.6	0.37	13	479
48	PC	W	NA	5.30	7	7.2	4	0.2	0.8	0.40	15	467
48	UC	W	AI	5.83	8	3.4	6	0.8	0.5	0.43	25	513
48	PC	W	AI	6.27	8	2.6	7	0.8	0.6	0.45	34	500
			s.e.m.$	0.287	8.4	2.31	2.6	0.85	1.23	0.045	11.0	17.2
Levels of significance												
Wilt				***	***	*	***	***	***	***	***	***
Chop				*	NS	*	NS	NS	*	NS	**	NS
Compaction				*	NS	NS	NS	NS	NS	NS	NS	NS
Air infiltration				***	***	NS	NS	NS	***	NS	***	***
Wilt x chop				NS	NS	NS	NS	NS	*	NS	NS	*
Wilt x compaction				NS	NS	NS	NS	NS	NS	NS	NS	NS
Wilt x air infiltration				***	***	***	NS	NS	***	*	***	***
Chop x compaction				NS	NS	NS	NS	NS	NS	NS	NS	NS
Chop x air infiltration				NS	NS	NS	NS	NS	NS	NS	*	NS
Compaction x air infiltration				*	NS	NS	**	NS	NS	NS	NS	*
Wilt x chop x compaction				NS	NS	NS	NS	NS	NS	NS	NS	NS
Wilt x chop x air infiltration				NS	NS	NS	NS	NS	NS	NS	NS	NS
Wilt x compaction x air infiltration				NS	NS	*	*	NS	NS	NS	NS	NS
Chop x compaction x air infiltration				NS	NS	NS	NS	NS	NS	NS	NS	NS
Wilt x chop x compaction x air infiltration$				NS	NS	NS	NS	NS	NS	NS	NS	NS

UC = unchopped, PC = precision-chopped; NW = no compression weight, W = compression weight; NA = no air infiltration, AI = air infiltration; LA = lactic acid, Eth = Ethanol, AA = acetic acid, PA = propionic acid, BA = butyric acid, L: FP = proportion of lactic acid to other fermentation products (g/g), NH_3-N = ammonia-N (g/kg N), BC = buffering capacity (m. Eq/kg DM); NS = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, $ s.e.m. relates to 4 factor interaction

Table 3.3.4: Treatment effects on silage microbial composition (log_{10} cfu/g silage)

Treatment				Lactic acid bacteria	**Enterobacteria**	*Clostridia*	*Bacilli*	Yeast
Wilt (h)	Chop	***Compaction***	Air					
0	UC	NW	NA	5.8	0.3	2.3	1.5	3.8
0	PC	NW	NA	4.8	1.8	1.6	2.5	4.2
0	UC	NW	AI	5.5	2.0	5.2	5.0	3.8
0	PC	NW	AI	5.9	2.7	4.6	4.5	3.6
0	UC	W	NA	5.7	0.7	2.3	2.6	3.6
0	PC	W	NA	5.5	0.0	1.6	2.3	3.7
0	UC	W	AI	5.9	1.1	4.7	3.5	2.7
0	PC	W	AI	5.8	2.4	4.0	3.2	3.8
24	UC	NW	NA	5.9	1.2	1.8	2.6	3.7
24	PC	NW	NA	5.1	0.0	1.9	2.9	2.8
24	UC	NW	AI	6.7	4.2	3.0	5.4	5.6
24	PC	NW	AI	7.1	4.8	3.7	5.8	5.5
24	UC	W	NA	5.6	1.4	1.4	2.9	3.3
24	PC	W	NA	5.6	1.5	1.8	2.6	3.4
24	UC	W	AI	6.6	4.3	3.2	5.1	4.9
24	PC	W	AI	6.9	3.7	2.3	4.4	5.6
48	UC	NW	NA	4.9	0.9	1.9	3.3	4.7
48	PC	NW	NA	4.8	0.0	1.9	3.4	4.9
48	UC	NW	AI	7.6	2.5	2.3	5.6	5.8
48	PC	NW	AI	7.2	2.7	2.2	4.7	6.3
48	UC	W	NA	4.8	0.9	2.1	2.6	4.5
48	PC	W	NA	4.7	0.4	2.5	3.1	4.8
48	UC	W	AI	6.3	1.8	2.2	3.3	6.4
48	PC	W	AI	7.0	2.3	2.2	4.0	6.5
			s.e.m.$	0.19	0.73	0.24	0.29	0.37
Levels of significance								
Wilt				***	**	***	***	***
Chop				NS	NS	NS	NS	NS
Compaction				NS	NS	NS	***	NS
Air infiltration				***	***	***	***	***
Wilt x chop				NS	NS	*	NS	NS
Wilt x compaction				**	NS	*	NS	NS
Wilt x air infiltration				***	NS	***	**	***
Chop x compaction				*	NS	NS	NS	NS
Chop x air infiltration				**	NS	NS	NS	NS
Compaction x air infiltration				*	NS	*	***	NS
Wilt x chop x compaction				NS	NS	NS	**	NS
Wilt x chop x air infiltration				NS	NS	NS	NS	NS
Wilt x compaction x air infiltration				NS	NS	NS	NS	NS
Chop x compaction x air infiltration				NS	NS	NS	NS	NS
Wilt x chop x compaction x air infiltration$				**	NS	NS	NS	NS

UC = unchopped, PC = precision-chopped; NW = no compression weight, W = compression weight; NA = no air infiltration, AI = air infiltration; NS = not significant, * = P < 0.05, ** = P < 0.01, *** = P < 0.001, $ s.e.m. relates to 4 factor interaction

Table 3.3.5: Treatment effects on silage aerobic stability, fresh weight loss and compaction variables

Wilt (h)	Chop	*Compaction*	Air	Interval (h) to temp. rise >2 °C	Max temp. rise (°C)	Interval (h) to max temp. rise	ACT 120 (°C)	Fresh weight loss (g/kg)	Silage height (cm)	Silage pore space (proportion)
0	UC	NW	NA	37	20	50	26	12	44	0.51
0	PC	NW	NA	33	16	46	27	13	38	0.44
0	UC	NW	AI	21	11	61	37	25	40	0.42
0	PC	NW	AI	29	14	69	38	32	32	0.35
0	UC	W	NA	43	21	54	31	13	31	0.31
0	PC	W	NA	49	15	66	23	12	27	0.29
0	UC	W	AI	115	7	131	12	48	29	0.33
0	PC	W	AI	77	10	104	14	64	28	0.30
24	UC	NW	NA	192	1	192	4	8	26	0.70
24	PC	NW	NA	192	0	192	1	7	22	0.65
24	UC	NW	AI	57	4	118	13	39	23	0.67
24	PC	NW	AI	96	4	128	12	44	22	0.65
24	UC	W	NA	192	1	192	3	8	22	0.64
24	PC	W	NA	192	1	192	2	9	22	0.65
24	UC	W	AI	151	5	171	16	44	19	0.61
24	PC	W	AI	133	4	181	5	37	19	0.60
48	UC	NW	NA	168	1	175	0	9	24	0.78
48	PC	NW	NA	192	0	192	1	15	23	0.77
48	UC	NW	AI	171	2	192	3	96	22	0.77
48	PC	NW	AI	137	2	101	7	64	20	0.74
48	UC	W	NA	192	0	192	5	17	22	0.76
48	PC	W	NA	192	0	192	4	13	20	0.75
48	UC	W	AI	192	1	192	7	48	20	0.75
48	PC	W	AI	165	3	124	9	56	18	0.75
			s.e.m.$	24.2	1.6	21.4	3.6	5.7	0.7	0.014
Levels of significance										
Wilt				***	***	***	***	***	***	***
Chop				NS	NS	NS	NS	NS	***	***
Compaction				**	NS	*	*	NS	***	***
Air infiltration				**	NS	NS	*	***	***	***
Wilt x chop				NS	NS	NS	NS	NS	***	*
Wilt x compaction				NS	NS	NS	***	***	***	***
Wilt x air infiltration				***	***	*	*	***	NS	NS
Chop x compaction				NS	NS	NS	NS	NS	***	**
Chop x air infiltration				NS	*	NS	NS	NS	NS	NS
Compaction x air infiltration				*	NS	NS	**	NS	NS	*
Wilt x chop x compaction				NS	NS	NS	NS	NS	*	NS
Wilt x chop x air infiltration				NS	NS	NS	NS	NS	NS	NS
Wilt x compaction x air infiltration				NS	NS	NS	*	***	***	***
Chop x compaction x air infiltration				NS	NS	NS	NS	NS	NS	NS
Wilt x chop x compaction x air infiltration$				NS	NS	NS	NS	*	NS	NS

UC = unchopped, PC = precision-chopped; NW = no compression weight, W = compression weight; NA = no air, AI = air infiltration; ACT 120 = Accumulated temperature rise to 120 h; NS = not significant, * = P < 0.05, ** = P < 0.01, *** = P < 0.001, $ s.e.m. relates to 4 factor interaction

Experiment 3.4. Bacterial community dynamics during the ensilage of wilted grass

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This study examined the effects of dry matter concentration and air infiltration on bacterial community composition during ensilage. A culture-independent technique, T-RFLP, was employed along with traditional methods of silage analysis, to assess the effect of these factors on microbial community composition and final silage quality.

Materials & methods: The forage treatments were organised in a 2 (ensiling system) x 6 (stage of ensiling) factorial arrangement. Herbage (303 ± 30.2 g dry matter/kg) was ensiled after a 24 h wilting period in cylindrical bales or as precision-chopped grass in laboratory silos. Three replicates of each treatment were sampled after 0, 2, 6, 14, 35 and 98 days of ensiling. Specific bacteria and yeast were enumerated and fermentation variables were assessed at each sampling time. Total silage bacterial DNA was extracted and T-RFLP was applied to the herbage samples to generate a community fingerprint.

Herbages were produced and ensiled as described in Experiment 3.3. Briefly, an homogenous plot of *Lolium perenne* was mown, wilted in the field for 24 h, following which representative samples of the herbage were baled with a round baler (Claas 250 Rotacut, chopping knifes disengaged) into fifteen 1.2 m wide x 1.2 m diameter cylindrical bales and wrapped in 6 layers of polythene stretch-film (750 mm wide, 0.025 mm thickness pre-stretching, 1.70 stretch). The wrapped bales were stored outdoors under protection from wildlife. At the time of baling, fifteen representative samples of wilted herbage were obtained from across the field. They were precision-chopped (Mex VI, Pottinger; 19 mm nominal chop length) and ensiled in units of 4.7 kg chopped herbage in plastic pipe silos (height = 0.75 m, internal diameter = 0.152 m, internal volume = 13.6 l). Laboratory silos were stored at a room temperature of 15 °C prior to sampling.

Three representative samples of herbage were taken after field wilting to characterise the grass prior to ensiling (day 0). Triplicate bales and laboratory silos were destructively sampled after 2, 6, 14, 35 and 98 days ensilage. Bales were aseptically sampled, at eight representative points around the bale using an electrically powered cylindrical core bit (length = 65 cm, internal diameter = 3.5 cm) and these eight core samples were composited to produce one sample per bale. Laboratory silos were opened and, after thorough aseptic mixing, one sample per silo was taken. Grass and silage samples were stored at 4 °C prior to microbiological analyses and at – 18 °C prior to chemical and molecular analyses.

Results: Silage pH ($P<0.05$), propionic acid concentration ($P<0.01$) and water soluble carbohydrate concentration ($P<0.01$) were all lower, while lactic acid bacterial numbers were higher ($P<0.01$) in the precision-chop silage compared to the baled silage (Table 1). Herbage pH and water soluble carbohydrate concentration decreased ($P<0.001$), while the concentration of fermentation products and ammonia-N all increased ($P<0.001$), over the course of fermentation in both ensiling systems. The greatest increase in lactic acid, acetic acid, propionic acid and ethanol occurred during the first 2 days of ensilage. Butyric acid concentration was low, only increasing ($P>0.05$) towards the end of the sampling period.

Stage of ensiling had a significant effect on herbage microbial composition as determined by enumeration using culture based approaches (Table 1). The numbers of lactic acid bacteria increased ($P<0.001$) rapidly in both systems after ensiling with the highest numbers observed on day 14 of ensiling, and then decreasing through to day 98. *Enterobacteria* numbers peaked on day 2 and then declined ($P<0.001$) over the remainder of the sampling period, and were not detected on day 98 of ensilage. Yeast numbers were also greatest on day 2 of ensiling before declining ($P<0.001$), but with numbers increasing again towards the end of the storage period. Numbers of *Clostridia* increased ($P<0.01$) at a low level from day 6 onward.

The interaction of ensiling system and stage of ensiling had little significant effect on the variables measured (Table 1). Lactic acid bacteria numbers peaked on day 14 in precision-chop silage at a higher level ($P<0.01$) than in baled silage. Changes in propionic acid concentration, although statistically significantly ($P<0.05$), were relatively minor as the concentration was relatively low (< 2.5 g kg^{-1} dry matter) in all silages.

T-RFLP was used to investigate the dynamics of the microbial community during fermentation, and for each sample a profile was obtained consisting of a range of TRFs present and their relative abundances. A total of seventy eight distinct TRFs were detected after analyses of all samples. Fifty three distinct TRFs were detected in the baled silages over the course of the fermentation compared to seventy three for the precision-chop silages, with five TRFs unique to the baled silages and twenty five TRFs unique to the precision-chop silages. However, total mean ribotype numbers did not vary markedly between ensiling systems over the course of the fermentation, ranging from 15.0 ± 9.0 to 20.3 ± 13.7 for the baled and precision-chop silages at each time point, respectively.

Changes in percentage relative abundance of the five most abundant ribotypes over the course of the fermentation in baled and precision-chop silages are illustrated in Figures 1a and 1b. Although, the top five most abundant TRFs differed for each ensiling system, there were three TRFs common to both systems (TRF 554, TRF 71 and TRF 493). In both systems, TRF 554 rapidly became the most abundant ribotype after ensiling, while in contrast TRF 493 decreased in relative abundance. In baled silage TRF 577 also increased in relative abundance after ensiling, peaking on day 6,

while an increase in abundance was observed for TRFs 71 and 72 to day 35 before a decrease occurred. In the precision-chop silage an increase in relative abundance was observed for TRFs 71 and 580 to day 35 followed by a decrease to day 98. Also between these two latter stages of ensiling, TRF 574 became the most abundant TRF in the precision-chop silage in contrast to TRF 554 for baled silage.

An MDS ordination plot was used to compare and visualise gross differences in bacterial community structure between ensiling systems over the course of the fermentation, for the top 20 most abundant TRFs which represented 89.6 % of overall abundance (Figure 2). Similarity between samples is presented as distance measures on an MDS ordination plot, so that ordinations are typically interpreted based on the distance between ordinate points. Thus, where sample points or treatments (e.g. triplicates for a particular silage sampling time) appear close together, these can be regarded as having a similar bacterial community composition. The stress value (0.11) indicated that the MDS ordination plot was a reliable spatial representation of the differences within the data. The largest difference in community composition typically occurred between day 0 samples and those from day 98. However, while the plot revealed a marked shift in community structure over the course of the fermentation (visualised as a left to right gradation across the plot), there was relatively little difference between the two ensiling systems. In both ensiling systems, community structure changed after ensiling (from day 0) as the fermentation progressed, remaining similar for days 2, 6, 14 and 35 of ensilage, but with a final shift in the community structure with extended storage to day 98.
While MDS analysis gives a broad view of treatment effects on bacterial community composition, a further multivariate statistical approach was carried out in order to elucidate the effects of the main ensilage factors on community composition. As the top 20 most abundant TRFs accounted for the majority of the variance in the data-set only these TRFs were used for subsequent analyses. Figure 3 shows a redundancy analysis (RDA) ordination plot of the top 20 most abundant TRFs in relation to ensiling system, stage of ensiling, the interaction between ensiling system (system) and stage of ensiling (stage), lactic and acetic acid concentrations, pH, ammonia-N (NH_3-N) and water soluble carbohydrate concentrations (WSC). Axes 1 ($P<0.01$) and 2 were found to explain 35.3 % and 6.6 % of the overall variance within the TRF data, respectively, accounting for 42 % of the total variance. The cumulative species-environment relation for Axes 1 and 2 was 85 %, indicating that these axes accounted for the bulk of the variance in the TRF data that could be attributed to ensilage factors (e.g. stage of ensiling, ensiling system and pH). Species-environment correlations for both axes were above 0.68, indicating that the TRF data were strongly correlated with ensilage parameters. Canonical coefficients and intraset correlations for the ensilage factors for each axis indicated that Axis 1 was primarily a lactic acid gradient and Axis 2 a ammonia-N gradient. Monte-Carlo significance tests indicated that both axes explained a significant proportion ($P<0.01$) of the variation in the data.
In general on the RDA ordination plot, the direction of the arrows for individual ensilage factors indicates an increasing concentration of that factor and the magnitude of the arrows determines the relative importance of that factor or interaction. The position of the ribotypes in relation to the factor arrows indicates how a particular ribotype is influenced and correlates to a given ensilage factor/interaction. These data indicate that the stage of ensiling, the concentrations of lactic acid, water soluble carbohydrate and ammonia-N and pH all influenced microbial community structure. As an example, the position of TRFs 493 and 480, located in the same direction as the arrow for pH and in the opposite direction to the arrow for lactic acid concentration, indicates that these fragments were negatively correlated with increasing lactic acid concentration and positively correlated with increasing pH. This indicates that these TRFs decrease in abundance over the course of the fermentation as lactic acid concentration increases and pH decreases. The ordination plot also suggests that two potentially different groups of putative lactic acid bacteria exist in the community. The larger group, represented by TRFs 554 and 577, appear to be influenced strongly by all the ensilage factors as their positions are located away from these factors on the ordination plot. This may reflect changes in their relative abundances over the course of the fermentation in response to chemical changes. The second group, represented by TRFs 574 and 584, are positively correlated with stage of ensiling reflecting their increase in abundance late in the ensilage period independently of other factors such as pH and lactic acid concentration.
TRF data was analysed further by ANOVA, which indicated the effect of the main factors (ensiling system, stage of ensiling) on specific individual ribotypes in the top twenty most abundant TRFs across all samples (Table 2). TRF 554 was the most abundant ribotype detected, followed by TRFs 71 and 493. These three ribotypes were present in the top five most abundant ribotypes detected in both ensiling systems. The stage of ensiling (and associated chemical changes) had the greatest significant effect on the top 20 TRFs, while the ensiling system had a significant effect on only 4 of the top 20 TRFs (TRFs 580, 586, 581 and 75). TRFs 580 and 586 were more abundant ($P<0.001$) in the precision-chop silage, while the opposite was the case for TRFs 581 and 75. Stage of ensiling had a positive effect on TRFs 554, 71, 577, 574, 581 and 612 with the ribotype abundance increasing ($P<0.001$) after ensiling or at some stage during the fermentation. In contrast, one of the most abundant ribotypes, TRF 493, decreased ($P<0.001$) in abundance over the course of fermentation, as mentioned previously. Several trends indicated by the RDA ordination plot are

supported by the ANOVA results. For example, the stage of ensiling and associated chemical changes had a significant positive effect on TRF 574 and a significant negative effect on the abundance of TRF 493.

The TAP-TRFLP database was used to assign putative phylogenetic identities based on TRF size after an *in-silico* digest with restriction enzyme *Msp*I. The database suggested a number of possible identities for each TRF. TRF 554 could possibly represent a group containing lactic acid bacteria species including *Streptococcus sp*., *Lactobacillus sp*., *Leuconostoc sp*., and also *Bacilli sp*. TRF 493 was a possible match for a larger group (132 possible identities) containing mainly *Enterobacteria sp*. including *Klebsiella sp*., *Escherichia sp*. and *Erwinia sp*. TRFs 71 and 72 were represented as mainly uncultured organisms in the database but did include *Streptococcus sp*., *Enterococcus sp*. and *Clostridia sp*. Other ribotypes from the top 10 most abundant TRFs, including TRFs 577, 580, 574, 584 and 581 could have possible matches with *Lactobacillus sp*. and *Pediococcus sp*, while TRF 586 was present as an uncultured organism.

All the clones sequenced from days 2 and 6 of ensiling from the baled silage system belonged to the lactic acid bacteria group (Table 3). After 2 days ensiling, the most readily identified bacteria (> 98 % homology) included *Leuconostoc mesenteroides*, *Leuconostoc carnosum*, *Lactobacillus sakei* and uncultured *Lactobacillus sp.* with terminal fragments (based on a simulated digest using restriction enzyme *Msp*I) of 555 and 612, 555, 578 and 578 bp, respectively. The *Leuconostoc carnosum* and *Lactobacillus sakei* sequences were common to samples from day 2 and day 6 of ensiling. Further sequences, obtained from samples on day 6 of ensiling, identified *Lactobacillus graminis* and another *Leuconostoc mesenteroides* strain with two terminal fragments of 555 and 612 bp for the latter sequence and one fragment of 555 bp for the former.

Conclusion: In this experiment, good quality silages were produced under controlled conditions and little difference in silage quality and microbial composition were observed between ensilage systems. T-RFLP proved a potentially useful tool to study the ensilage process and for following the dynamics of bacterial community changes over the course of the fermentation, and generally agreed with findings from conventional analyses. In such a copiotrophic environment, where a restricted group of microorganisms are responsible for community changes, it is possible that culture-based approaches are adequate tools to study the changes in microbial composition. However, the use of culture-independent approaches could potentially result in the analysis of a greater extent of the community (e.g. uncultured *Lactobacillus sp*. were detected by cloning). Allied to this the rapid nature of some molecular methods, the range of methods available and the ability to target different microbial groups using specific primers shows the great potential of DNA-based methods in the analysis of complex microbial systems. Furthermore, a significant benefit of the T-RFLP method is that an overview of the whole bacterial population can be obtained in one assay. As a result, T-RFLP offers a new method to study the ensilage process and offers a viable alternative to culture-dependent methods.

Table 3.4.1: Interactions between ensiling system and stage of ensiling – differences in silage pH, fermentation products (g/kg dry matter, unless otherwise stated), water soluble carbohydrate concentration (g/kg dry matter) and microbial composition (log_{10} cfu/g herbage)

Treatment			Fermentation products							Microbial composition			
Ensiling system	Stage of ensiling (d)	pH	LA	AA	Eth	BA	PA	NH_3-N (g/kg N)	WSC	LAB	*Enterobacteria*	*Clostridia*	Yeast
BS	0	6.33	2	2	1	0.4	0.3	12	178	4.3	2.9	1.3	1.8
BS	2	5.65	50	14	10	0.2	1.9	20	125	7.9	5.8	1.4	3.2
BS	6	4.79	44	7	13	0.6	1.4	30	71	8.0	4.5	1.3	0
BS	14	4.58	55	9	17	0.7	2.4	43	33	8.2	2.1	2.5	0.1
BS	35	4.28	61	13	13	0.7	1.3	43	20	7.7	0.3	1.9	1.5
BS	98	4.03	72	12	12	1.4	0.7	60	26	5.9	0	2.4	2.3
PS	0	6.33	2	2	1	0.4	0.3	12	178	4.3	2.9	1.3	1.8
PS	2	5.40	48	15	8	1.1	1.6	18	101	8.1	5.7	1.0	4.0
PS	6	4.67	46	9	11	0	0.8	28	41	8.1	3.2	1.4	0.5
PS	14	4.42	52	8	13	0.3	1.1	37	18	9.4	0.7	1.7	0.8
PS	35	4.23	47	10	13	1.9	1.4	37	14	7.8	0.9	2.2	1.9
PS	98	3.98	67	14	11	2.6	0.6	56	12	6.0	0	2.2	1.4
	s.e.m.†	0.078	5.0	1.3	1.5	0.71	0.22	5.2	7.7	0.15	0.59	0.36	0.28
Levels of significance													
Ensiling system		*	NS	NS	NS	NS	**	NS	**	**	NS	NS	NS
Stage of ensiling		***	***	***	***	***	***	***	***	***	***	**	***
Ensiling system x stage of ensiling†		NS	NS	NS	NS	NS	*	NS	NS	**	NS	NS	NS

BS = baled silage, PS = precision-chop silage; LA = lactic acid, AA = acetic acid, Eth = ethanol, BA = butyric acid, PA = propionic acid, NH_3-N = ammonia-N; WSC = water soluble carbohydrate; LAB = lactic acid bacteria; †s.e.m. relates to the interaction between ensiling system and stage of ensiling, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, NS = not significant.

Figures 3.4.1a – 1b: Changes in the relative abundance of the top five most abundant TRFs over the course of the sampling period in baled and precision-chop silages.

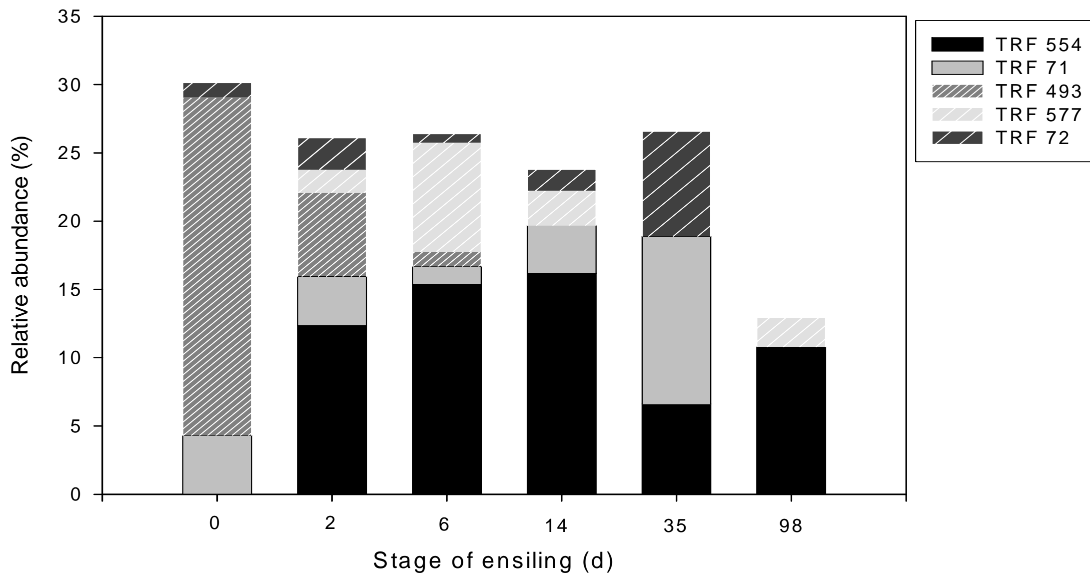


Fig. 3.4.1a: Baled silage

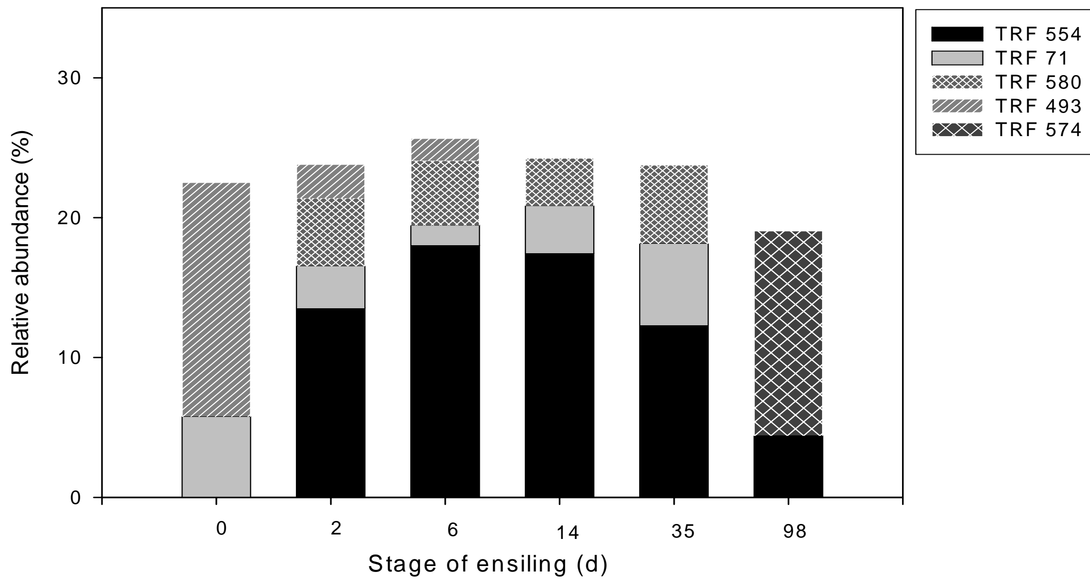
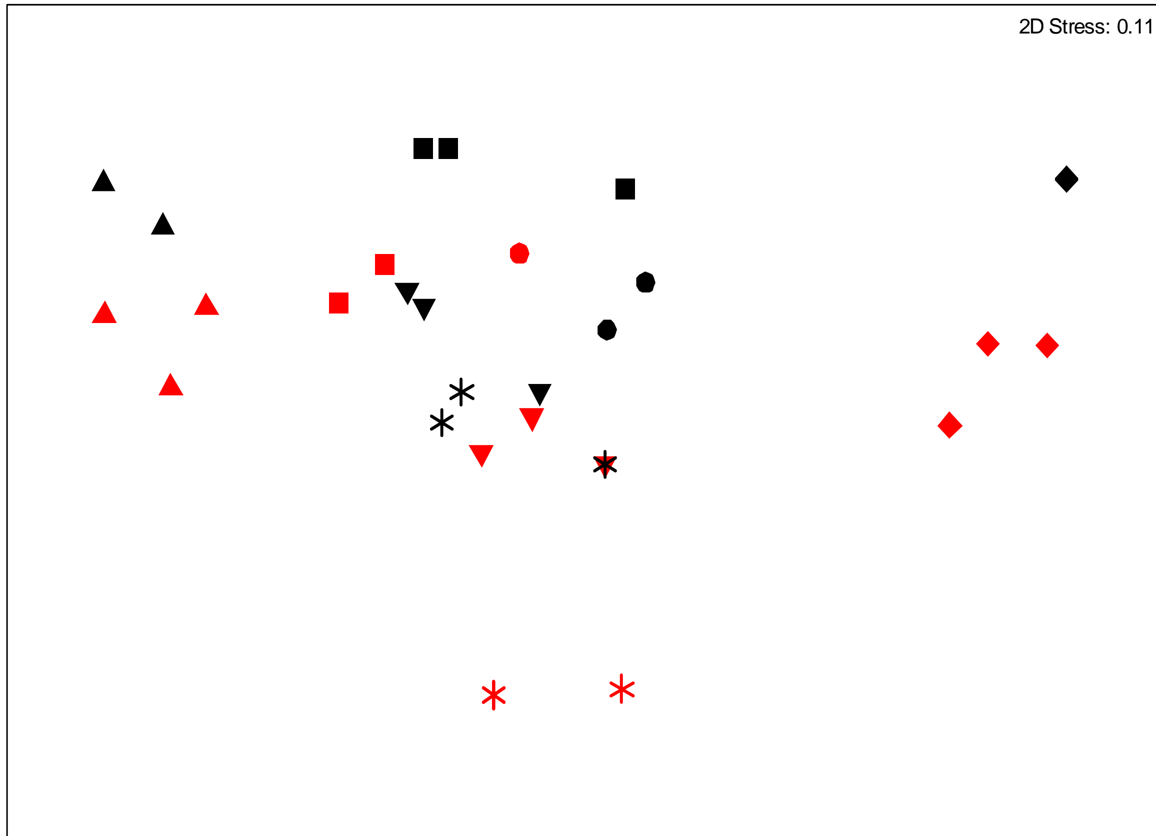


Fig. 3.3.1b: Precision-chop silage

Figures 3.4.2: Multidimensional scaling (MDS) ordination diagram of T-RFLP data (top 20 most abundant TRFs) on days 0 (▲), 2 (■), 6 (●), 14 (▼), 35 (*) and 98 (♦) of ensiling in baled (red symbols) and precision-chop (black symbols) silages.



Note: symbols may overlap if sample community composition is similar.

Figure 3.4.3: Redundancy analysis (RDA) ordination diagram of T-RFLP data, with explanatory (e.g. ensiling system, lactic acid concentration) variables represented as large arrows and TRFs as small triangles. TRFs are labelled according to fragment size (bp). TRFs representing lactic acid bacteria species, based on putative identification using the TAP-TRFLP database, are presented in red. WSC = water soluble carbohydrate concentration, NH_3-N = ammonia-N concentration.

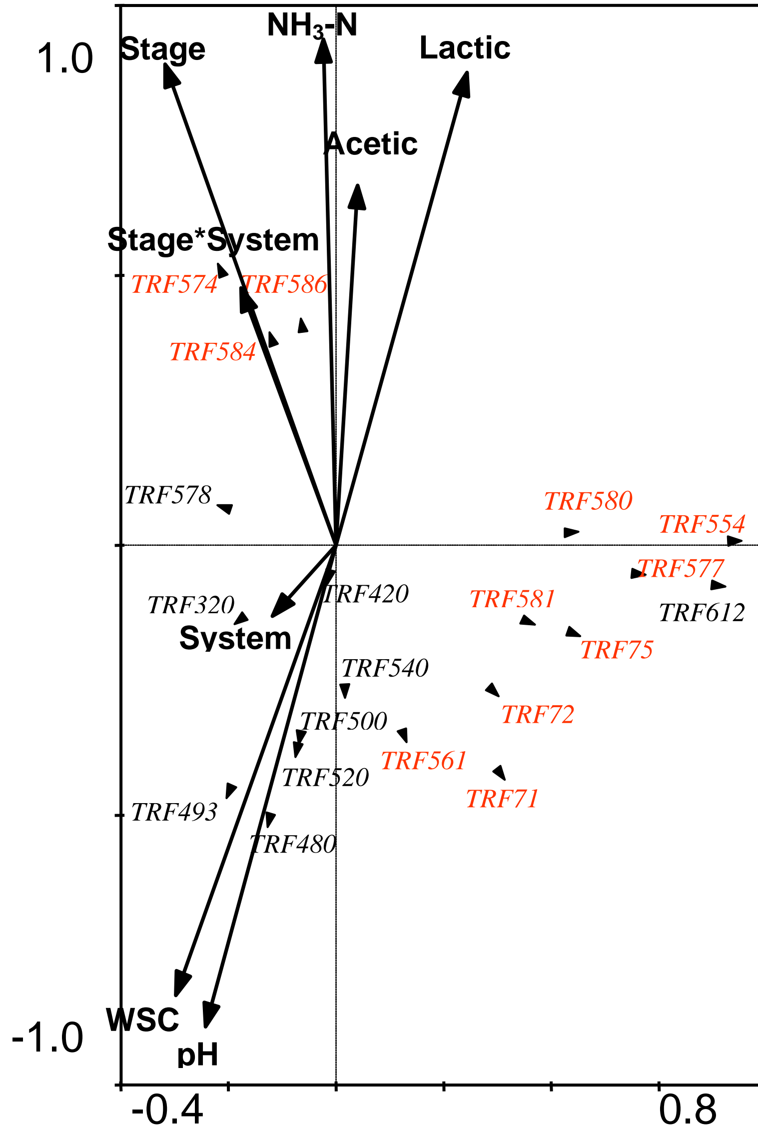


Table 3.4.2: ANOVA results for the top 20 terminal restriction fragments (TRFs) in the T-RFLP data, as ranked by abundance

TRF size (bp)	Abundance rank	% Abundance	Cumulative % abundance	*P-values* Ensiling system	Stage of ensiling	Ensiling system x stage of ensiling
554	1	29.3	29.3	0.952	**<0.001**$^{+}$	0.536
71	2	11.6	40.9	0.145	**<0.001**$^{+}$	0.420
493	3	8.6	49.5	0.524	0.002^{-}	0.968
577	4	6.3	55.8	0.242	**<0.001**$^{+}$	0.144
72	5	5.7	61.4	0.286	**<0.001**	0.021
580	6	5.5	66.9	0.006^{+PS}	**<0.001**$^{-}$	**<0.001**
574	7	5.2	72.1	0.508	**<0.001**$^{+}$	0.808
586	8	3.3	75.4	**<0.001**$^{+PS}$	**<0.001**	**<0.001**
584	9	3.1	78.5	0.485	**<0.001**	0.770
581	10	2.3	80.8	**<0.001**$^{+BS}$	**<0.001**$^{+}$	**<0.001**
612	11	1.8	82.6	0.276	**<0.001**$^{+}$	0.474
75	12	1.3	83.9	0.011^{+BS}	0.007^{-}	0.239
578	13	1.1	85.0	0.166	0.109	0.109
561	14	0.9	85.9	0.903	**<0.001**$^{-}$	0.510
520	15	0.7	86.6	0.781	**<0.001**$^{-}$	0.457
500	16	0.6	87.2	0.987	**<0.001**$^{-}$	0.531
540	17	0.6	87.8	0.340	**<0.001**$^{-}$	0.217
480	18	0.6	88.4	0.561	**<0.001**$^{-}$	0.306
320	19	0.6	89.0	0.327	0.020	0.004
420	20	0.6	89.6	0.369	0.477	0.450

Bold indicates a significant effect ($P < 0.001$); $^{+}$ = positive effect on TRF abundance, $^{-}$ = negative effect on TRF abundance; ^{PS} = precision-chop silage, ^{BS} = baled silage.

Table 3.4.3: Identities of clones obtained by sequencing and BLAST analysis from day 2 and day 6 baled silages.

Stage of ensiling (d)	Closest relative (accession number)	% similarity to closest relative
2	*Leuconostoc mesenteroides* (CP000414.1)	99
2, 6	*Leuconostoc carnosum* (AB022925.1)	98
2, 6	*Lactobacillus sakei* (CR936503.1)	99
2	*Lactobacillus sp.* (FF590122.1)	99
6	*Lactobacillus graminus* (AM113778.1)	99
6	*Leuconostoc mesenteroides* (EF068254.1)	99

Experiment 3.5. Assessing the impacts of various ensilage factors on fermentation in grass silage using traditional microbiology culture and bacterial community analysis techniques

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This study compared traditional microbiology culture and bacterial community analysis techniques using herbage ensiled under contrasting DM, compaction and air infiltration conditions.

Material and methods: Two separate experiments were undertaken to study the effects of forage DM concentration in baled and conventional silages over the course of ensilage, and of air infiltration and compaction, on silage fermentation quality and bacterial community composition. The first experiment (3.5A was organised in a 2 (DM concentration) x 2 (ensiling system) x 4 (stage of ensiling) factorial arrangement of treatments. Herbage was ensiled after both a 0 and 48 h wilt period, corresponding to a low and high DM concentration, in cylindrical bales or as precision-chop grass in laboratory silos. Triplicate units of each ensiling treatment were sampled prior to ensiling and after 2, 6 and 14 days of

ensiling. The second experiment (3.5B) was organised in a 2 (compaction) x 2 (air infiltration) factorial arrangement of treatments. Herbage was ensiled unchopped, directly after harvesting in laboratory silos with or without compaction, and was stored with or without air infiltration for a period of 100 days. Specific bacteria and yeast were enumerated and fermentation variables were assessed pre and post ensilage. Total silage bacterial DNA was extracted and T-RFLP was applied to the herbage samples to generate a community fingerprint.

Experiment 3.5A: Herbages were produced as described in Experiment 3.2. Briefly, an homogenous plot of *Lolium perenne* was mown and field wilted. After 0 (185 ± 7.0 DM kg^{-1}) and 48 (406 ± 29.8 DM kg^{-1}) h field wilting, representative samples of the herbage were picked up with a round baler (Claas 250 Rotacut) and baled into nine 1.2 m wide x 1.2 m diameter cylindrical bales and wrapped in 6 layers of polythene stretch film (750 mm wide, 0.025 mm thickness pre-stretching, 1.70 pre-stretch). At the time of baling, representative samples of herbage were obtained from across the field for filling laboratory silos. Each laboratory silo was filled with 5.0 (0 h wilt; n = 9) or 4.1 kg (48 h wilt; n = 9) of herbage, with the herbage being precision-chopped (Pottinger, Mex VI; 19 mm nominal chop length) immediately prior to ensiling. The plastic pipe silos (height = 0.75 m, internal diameter = 0.152 m, internal volume = 13.6 l), were packed manually by a repeatable procedure, and sealed mechanically by a screw-on base and lid with rubber seals. Compaction was achieved by the inclusion of a 10.5 kg weight (5.83 kPa continual vertical pressure) directly onto the herbage and each lid was fitted with a gas release valve. Laboratory silos were stored at a room temperature of 15 °C prior to sampling.

Three representative herbage samples for each treatment were taken prior to ensiling (day 0). Triplicate bales and laboratory silos were destructively sampled after 2, 6 and 14 days of ensilage for each DM treatment. Bales were aseptically sampled, at eight representative points around the bale using an electrically powered cylindrical core bit and these eight core samples were composited to produce one sample per bale. Laboratory silos were opened and after thorough aseptic mixing, one sample per silo was taken. All samples were stored at 4 °C prior to microbiological analyses and at – 18 °C prior to chemical and molecular analyses.

Experiment 3.5B: Herbages were produced as described in Experiment 3.3. Briefly, an homogenous plot of *Lolium perenne* (185 ± 4.7 DM kg^{-1}) was mown and representatively sampled. The sampled herbage (0.8 kg DM) was used to fill twelve laboratory pipe silos). The following treatments were applied: (1) compaction, no air infiltration (control), (2) compaction plus air infiltration, (3) no compaction, no air infiltration and (4) no compaction plus air infiltration. Compaction was achieved in these silos by the inclusion of a 21 kg steel weight directly onto the herbage, while the remainder had no weight included. Air infiltration was achieved by incomplete sealing, with the screw-on base and lid of these silos only being hand tightened while complete anaerobiosis was achieved by mechanically tightening and sealing the remainder. Laboratory silos were stored at 15 °C prior to sampling.

Three representative herbage samples were taken prior to ensiling (day 0). Laboratory silos were sampled after 100 days of ensilage.

Results: *Experiment 3.5A:* Wilting grass prior to ensiling had a significant effect on the ensilage process for both baled and precision-chop silages. pH was higher ($P<0.001$, see results of Experiment 3.2), while lactic acid, volatile fatty acids, ethanol and ammonia-N concentrations were all lower ($P<0.001$; see results of Experiment 3.2) in the silage prepared from herbage wilted to 406 g/kg DM (high DM). The higher concentration of fermentation products in the silages prepared from the 185 g DM /kg herbage (low DM) was reflected in a lower ($P<0.01$) concentration of residual water soluble carbohydrates (see results of Experiment 3.2). Overall, in the low DM herbage, lactic acid bacteria numbers were higher ($P<0.01$), while On average in the high DM herbages, the extent and rate of decline in pH and water soluble carbohydrate concentration decreased ($P<0.001$) over time relative to the low DM herbage. Lactic acid, volatile fatty acid and ammonia-N concentrations were higher ($P<0.001$) at each stage of ensiling in the low DM herbage (see results of Experiment 3.2). While lactic acid bacteria numbers were lower at ensiling (day 0) for the low DM herbage, the rate of increase after ensiling was greater and the numbers at each later sampling point were higher ($P<0.001$; see results of Experiment 3.2) in the low DM compared to the high DM herbage. *Enterobacteria* and yeast numbers declined ($P<0.001$; see results of Experiment 3.2) after ensiling in the high DM herbage, but increased to day 2 before declining in the low DM herbage.

Ensiling system also effected the fermentation, with average silage pH and water soluble carbohydrate concentration being lower ($P<0.001$) and lactic acid concentration higher ($P<0.001$) in the precision-chop silage compared to the baled silage (see results of Experiment 3.2). Although average lactic acid bacteria, *Clostridia*, *Bacilli* and yeast numbers were similar in both systems, higher ($P<0.001$) *Enterobacteria* numbers were observed in baled silage (see results of Experiment 3.2).

On days 2, 6 and 14 of ensiling, pH and water soluble carbohydrate concentration were lower ($P<0.05$) on average in the precision-chop compared to the baled silages (see results of Experiment 3.2). The increase in *Enterobacteria* numbers after ensiling was higher ($P<0.001$; see results of Experiment 3.2) and the decline in numbers from day 2 was slower for baled compared to precision-chop silage. The decrease ($P<0.01$; see results of Experiment 3.2) in

Enterobacteria numbers was faster in the low DM precision-chop silage, while numbers persisted at a high level in the high DM baled silage. In the high DM herbage, *Clostridia* numbers remained static over the course of the fermentation, while in contrast *Clostridia* numbers increased (P<0.001; see results of Experiment 3.2) in the low DM baled silage from day 2 of ensiling.

T-RFLP was used to investigate the dynamics of the microbial community during fermentation in low and high dry DM herbages ensiled in baled and precision-chop silage systems. A profile was obtained for each sample consisting of a range of TRFs present and their relative abundances. Total mean ribotype numbers did not vary between treatments over the course of the fermentation, ranging between 6.5 ± 1.5 and 5.6 ± 3.0 for the low and high DM herbages at each time point, respectively. Overall, a total of 29 distinct TRFs were detected after analysis of all samples, with the number varying between DM treatments. A total of seventeen TRFs were detected in the low DM herbages over the course of the fermentation compared to twenty six for the high DM herbages, with three TRFs unique to the low and twelve TRFs unique to the high DM herbages, respectively. The top five most abundant ribotypes (% abundance) differed for each DM treatment and included TRFs 577 (24.5 %), 554 (13.5 %), 580 (12.0 %), 493 (11.1%) and 76 (10.4 %) in the low DM herbage, and TRFs 493 (44.8 %), 554 (11.8 %), 553 (8.3 %), 76 (7.2 %) and 580 (4.4 %) in the high DM herbage.

MDS ordination plots were used to compare and visualise gross differences in bacterial community structure, for the top twenty most abundant TRFs representing 99.2 % of overall abundance, over the course of the fermentation for the low and high DM herbages ensiled as baled and precision-chop silage (Figure 3.5.1a – 3.5.1b). Similarity between samples is presented as distance measures on an MDS ordination plot, so that ordinations are typically interpreted based on the distance between ordinate points. Thus, sample points that appear close together can be regarded as having a similar bacterial community composition. The stress value for Figure 3.5.1a (0.08) and 3.5.1b (0.10) indicated that the MDS ordination plots were a reliable spatial representation of the differences within the data. In general, the largest difference in community composition occurred between day 0 samples and those from day 2 or day 6 of ensiling. With the exception of the samples from day 14 of the precision-chop silages, a separation of the samples based on DM concentration was observed on both ordination plots. While the plots revealed a marked shift in community structure relative to DM concentration and stage of ensiling, there was relatively little difference detected between the two ensiling systems used. However, the shift in community structure during the ensilage of the high DM baled silage appeared less pronounced, with samples from day 0 and day 2 clustered more closely together than for the precision-chop silage.

A further multivariate statistical approach was carried out in order to elucidate effects of the main ensilage factors on community composition. Figure 3.5.2 shows a redundancy analysis (RDA) ordination plot of the top 20 most abundant TRFs in relation to DM concentration, ensiling system, stage of ensiling, the interaction of DM concentration and stage of ensiling, the concentrations of lactic acid, volatile fatty acid, ethanol, ammonia-N and water soluble carbohydrate, and silage pH. Axes 1 (P<0.01) and 2 were found to explain 32.1 % and 13.5 % of the overall variance within the TRF data, respectively, accounting for 45.6 % of the total variance. The cumulative species-environment relation for Axes 1 and 2 was 88.2 %, indicating that these axes accounted for the bulk of the variance in the TRF data that could be attributed to ensilage factors (e.g. DM, stage of ensiling). Species-environment correlations for both axes were above 0.73, indicating that the TRF data were strongly correlated with ensilage parameters. Canonical coefficients and intraset correlations for the ensilage factors for each axis indicated that Axis 1 was primarily a lactic acid gradient and Axis 2 a DM x stage of ensiling gradient. Monte-Carlo significance tests indicated that both axes explained a significant proportion (P<0.01) of the variation in the data.

In general, the direction of the arrows on the ordination plot for individual ensilage factors indicates an increasing concentration of that factor and the magnitude of the arrows determines the relative importance of that factor or interaction. The position of the ribotypes in relation to the factor arrows indicates how a particular ribotype is influenced and correlates to a given ensilage factor/interaction. These data would suggest that DM concentration and stage of ensiling heavily influenced microbial community structure whereas ensiling system had much less effect. The concentrations of lactic acid, water soluble carbohydrate and ammonia-N, and silage pH had less of an effect. For example, the position of TRFs 493 and 387, located in the opposite direction to the arrows for stage of ensiling, and lactic acid and volatile fatty acid concentration, indicates that these ribotypes were negatively correlated with time and the increasing concentration of fermentation products (i.e. these ribotypes decreased in abundance over the course of the fermentation). Increasing DM concentration was negatively correlated with a large group of TRFs including TRFs 577, 554, 76,115 and 573, and was also negatively correlated with other ensilage factors including lactic acid concentration, ammonia-N and pH.

These data were analysed further by ANOVA of the top 20 most abundant TRFs across all samples (Table 3.5.1), which indicated the effect of the main factors and their interactions on specific individual ribotypes. TRF 493 was the most abundant ribotype detected, followed by TRFs 577, 554, 76 and 580. Stage of ensiling (and the associated chemical

changes) had the greatest significant effect on the top 20 TRFs, followed by DM concentration, while ensiling system had a significant effect on only 3 of the top 20 TRFs (TRFs 553, 152 and 586). Stage of ensiling had a strong positive effect on TRFs 577, 554, 580 and 553 with the ribotype abundance increasing (P<0.001) after ensiling. In contrast, for the most abundant ribotype, TRF 493, a decrease (P<0.01) in relative abundance was observed over the course of the fermentation. DM concentration also had a strong positive effect on this ribotype (P<0.001) and to a lesser extent TRF 553 (P<0.01), with ribotype abundance being higher in the high DM herbage. The opposite was the case for TRFs 577 (P<0.001), 580 (P<0.01), 77 (P<0.01) and 586 (P<0.001), with a decrease in abundance observed in the high compared to the low DM herbages. TRFs 553 (P<0.01) and 586 (P<0.05) were more abundant in the precision-chop compared to the baled silage, while the opposite was the case for TRF 152 (P<0.01). Some trends indicated by the RDA ordination plot are supported strongly by the ANOVA results. For example, the stage of ensiling had a significant positive effect on TRF 586 and a significant negative effect on the relative abundance of TRF 493.

The TAP-TRFLP database was used to assign possible phylogenetic identities based on TRF size (bp) after an *in-silica* digest with restriction enzyme *Msp*I. The TAP database suggested a number of possible identities for each TRF which were narrowed down based on the groups of microorganisms expected in a silage fermentation. TRF 493 was a possible match for a large group (132 possible matches) containing mainly *Enterobacteria sp*. including *Klebsiella sp*., *Escherichia sp*. and *Erwinia sp*. TRF 577 was present in the database as an uncultured lactic acid bacterium, but was close to groups containing *Lactobacillus* and *Pediococcus sp*. (576 and 578 bp). TRFs 553 and 554 could possible represent a group containing mainly lactic acid bacteria species including *Streptococcus sp*., *Lactobacillus sp*., *Leuconostoc sp*., and also *Bacilli sp*, while TRFs 580, 581, 582, 586, 613 were also a possible match for groups containing *Lactobacillus sp*. and *Pediococcus sp*.

Experiment 3.5B: On average pH was lower (P<0.01) and ammonia-N concentration was higher (P<0.05) in the compacted silages, while pH (P<0.01) and numbers of *Clostridia* and *Bacilli* (P<0.001) were higher when air was allowed to infiltrate into the silos (Table 3.5.2). In the presence of air infiltration, *Bacilli* numbers were highest (P<0.001) in the uncompacted silage. Air infiltration and compaction had no further significant effects on indices of chemical and microbiological composition measured after 100 days ensilage.

Mean ribotype numbers did not vary between treatments, ranging from 11.3 ± 3.1 to 11.7 ± 4.0, 7.0 ± 1.0 and 6.3 ± 1.6 for treatments 1 - 4, respectively. An MDS plot, for the top 20 most abundant TRFs representing 96.4 % of the overall abundance, revealed a marked shift in community structure as a result of the compaction treatment; with samples from the uncompacted silages (treatments 3 and 4) located in the bottom half of the plot. When air infiltrated into the latter uncompacted silages a further shift in community structure was observed. The stress value (0.02) indicated that the MDS plot was a reliable spatial representation of the differences between the data. Samples from compacted silages (treatments 1 and 2) clustered together in the top half of the plot, regardless of the presence or absence of air infiltration indicating a close relationship between their respective community structures.

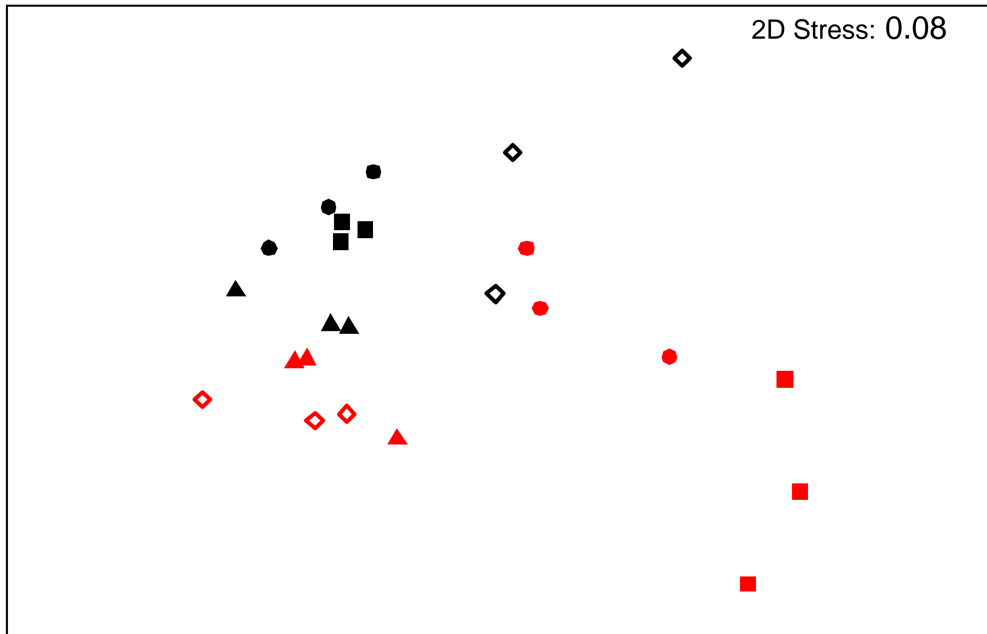
ANOVA indicated that the presence or absence of compaction had a greater effect on the top 20 most abundant TRFs than air infiltration (Table 3.5.3). The presence of compaction had a strong negative effect on TRF 180 (P<0.001) and to a lesser extent TRFs 499 (P<0.05) and 572 (P<0.01), with the ribotype abundance being lower in the presence of compaction. The opposite was the case for TRFs 221 (P<0.05), 220 (P<0.01), 155 (P<0.01) and 519 (P<0.01), with an increase in abundance observed in the presence of compaction. Air infiltration had a negative effect (P<0.01) on the abundance of TRFs 221 and 155.

Possible phylogenetic identities were once again assigned using the TAP-TRFLP database. This revealed that TRFs 180, 572 and 575 were a possible match for groups containing mainly *Lactobacillus sp*., TRFs 499 and 492 for groups containing mainly *Enterobacteria sp*., TRFs 220, 221, 517 and 519 for *Clostridium sp*. and TRF 155 for *Bacilli sp*., respectively.

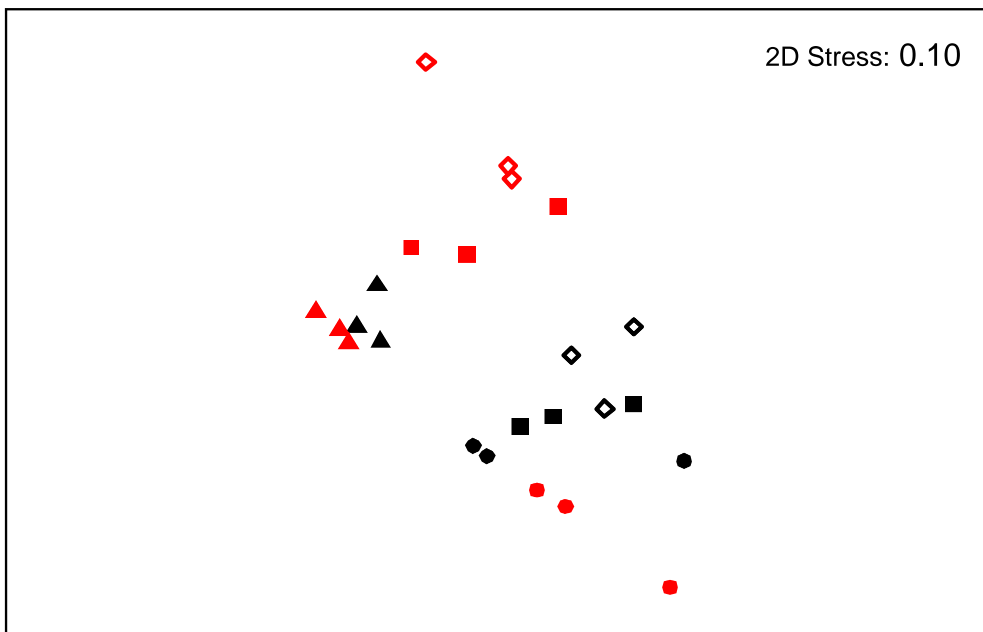
Conclusions: In general, both conventional microbiological and molecular data analyses did show similar shifts in bacterial community composition when contrasting ensiling treatments were compared. This change in community composition and the associated negative impacts on silage fermentation could be more pronounced in drier herbages where the fermentation would be more restricted and where air could be readily penetrate the silage mass.

A significant benefit of T-RFLP is that an overview of the whole bacterial community can be attained in one assay allowing for comparative community analysis between samples. T-RFLP can provide greater insight into the changes in bacterial community composition during ensiling, detecting subtle differences not evident through culturing and could provide an alternative method or a viable support tool to conventional methods used to study the ensilage process.

Figures 3.5.1a – 1b: Multidimensional scaling (MDS) ordination diagrams of T-RFLP data (top 20 most abundant fragments) for baled and precision-chop silages on days 0 (▲), 2 (◊), 6 (■) and 14 (●) of ensilage, in low dry matter (0 h wilt, black symbols) and high dry matter baled (48 h wilt; red symbols) herbages.



3.5.1a) Baled silage



3.5.1b) Precision-chop silage

Figure 3.5.2: Redundancy analysis (RDA) ordination diagram of T-RFLP data for the effect of dry matter concentration on community structure, with explanatory variables represented as large arrows and terminal restriction fragments (TRFs) as small triangles. TRFs are labelled according to fragment size (bp).

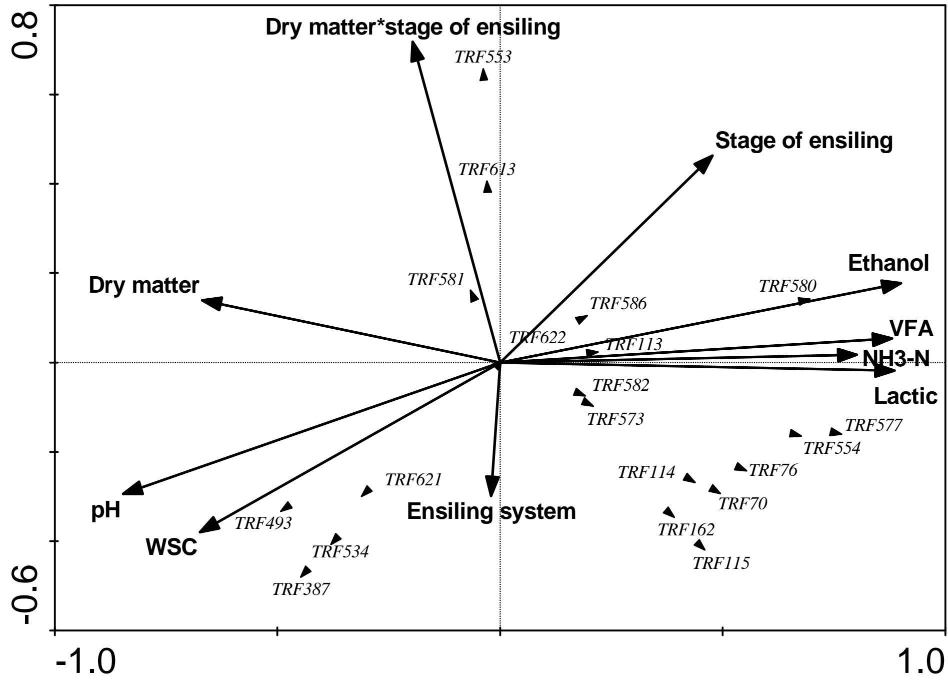


Table 3.5.1: ANOVA results for the top 20 terminal restriction fragments (TRFs), as ranked by abundance, for the effect of dry matter concentration on T-RFLP profiles.

				P-values						
TRF (bp)	Abundance rank	% Abundance	Cumulative % abundance	Dry matter	Ensiling system	Stage of ensiling	Dry matter x ensiling system	Dry matter x stage of ensiling	Ensiling system x stage of ensiling	Dry matter x ensiling system x stage of ensiling
493	1	28.0	28.0	**<0.001** $^{+}$	0.444	0.003 $^{-}$	0.058	0.344	0.708	0.106
577	2	12.7	40.7	**<0.001** $^{-}$	0.697	**<0.001** $^{+}$	0.251	**<0.001**	0.859	0.865
554	3	12.7	53.4	0.680	0.793	**<0.001** $^{+}$	0.119	0.493	0.458	0.370
76	4	8.8	62.2	0.140	0.619	0.022 $^{-}$	0.035	0.051	0.161	0.150
580	5	8.2	70.4	0.001 $^{-}$	0.211	**<0.001** $^{+}$	0.264	0.002	0.773	0.865
77	6	5.9	76.3	0.001 $^{-}$	0.144	0.006 $^{-}$	0.376	0.735	0.705	0.114
553	7	5.8	82.1	0.004 $^{+}$	0.004 $^{+PS}$	**<0.001** $^{+}$	**<0.001**	**<0.001**	**<0.001**	**<0.001**
114	8	5.0	87.1	0.043 $^{-}$	0.548	0.002 $^{-}$	0.112	0.110	0.428	0.213
115	9	2.7	89.8	0.279	0.521	**<0.001** $^{-}$	0.093	0.101	0.655	0.580
113	10	2.5	92.3	0.200	0.093	0.054	0.597	0.220	0.017	0.130
387	11	1.4	93.7	0.202	0.202	0.279	0.202	0.279	0.279	0.279
152	12	1.2	94.9	0.029 $^{-}$	0.009 $^{+BS}$	**<0.001** $^{-}$	**<0.001**	0.572	0.259	0.001
613	13	1.0	95.9	0.134	0.439	0.327	0.439	0.327	0.171	0.171
621	14	0.9	96.8	0.484	0.484	0.064	0.013	0.007	0.007	0.064
586	15	0.6	97.4	**<0.001** $^{-}$	0.022 $^{+PS}$	**<0.001** $^{+}$	0.269	**<0.001**	0.057	0.012
573	16	0.5	97.9	0.178	0.178	0.151	0.178	0.151	0.151	0.151
622	17	0.4	98.3	0.139	0.139	0.096	0.139	0.096	0.096	0.096
581	18	0.3	98.6	0.056	0.056	0.017 $^{+}$	0.056	0.017	0.017	0.017
582	19	0.3	98.9	0.081	0.081	0.035 $^{+}$	0.081	0.035	0.035	0.035
534	20	0.3	99.2	0.233	0.233	0.323	0.233	0.323	0.323	0.323

Bold indicates a strong significant effect ($P < 0.001$); $^{+}$ = positive effect on TRF abundance, $^{-}$ = negative effect on TRF abundance; ^{PS} = precision-chop silage, ^{BS} = baled silage.

Table 3.5.2: Grass composition (mean (SD)) prior to ensiling, and the effect of the interaction of compaction and air infiltration on pH, fermentation products (g/kg dry matter, unless otherwise stated), water soluble carbohydrate concentration (g/kg dry matter) and microbial composition ($\log_{10}$ cfu/g herbage).

Variable			pH	Fermentation products					WSC	Microbial composition				
				LA	AA	Eth	BA	NH_3-N (g/kg N)		LAB	*Enterobacteria*	*Clostridia*	*Bacilli*	Yeast
Grass (day 0)			6.13 (0.029)	-	-	-	-	-	171 (4.7)	4.2 (0.08)	4.1 (0.13)	1.6 (0.13)	2.6 (0.13)	2.5 (0.17)
Silage (day 100)														
Treatment	Compaction	Air infiltration												
1	C	NA	3.67	145	22	9.2	8.3	82	16	5.7	0.7	2.3	2.6	3.6
2	C	AI	3.87	134	23	10.1	8.4	76	16	5.9	1.1	4.7	3.5	2.7
3	NC	NA	3.77	151	36	5.1	3.4	58	17	5.8	0.3	2.3	1.5	3.8
4	NC	AI	4.43	148	22	4.6	3.9	63	16	5.5	0.2	5.2	5.0	3.8
		s.e.m.†	0.109	8.2	4.1	3.60	2.20	5.5	0.8	0.21	0.72	0.34	0.22	0.35
Levels of significance														
Compaction			*	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS
Air infiltration			**	NS	NS	NS	NS	NS	NS	NS	NS	***	***	NS
Compaction x air infiltration†			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	***	NS

C = compaction, NC = no compaction; NA = no air infiltration, AI = air infiltration; LA = lactic acid, AA = acetic acid, Eth = ethanol, BA = butyric acid, NH_3-N = ammonia nitrogen; WSC = water soluble carbohydrate; LAB = lactic acid bacteria; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, NS = not significant; † = s.e.m. relates to the interaction between compaction and air infiltration.

Figure 3.5.3: Multidimensional scaling (MDS) ordination diagram of T-RFLP data (top 20 most abundant fragments) of treatments 1 (□), 2 (●), 3 (■) and 4 (▲), for the effect of compaction and air infiltration on bacterial community structure.

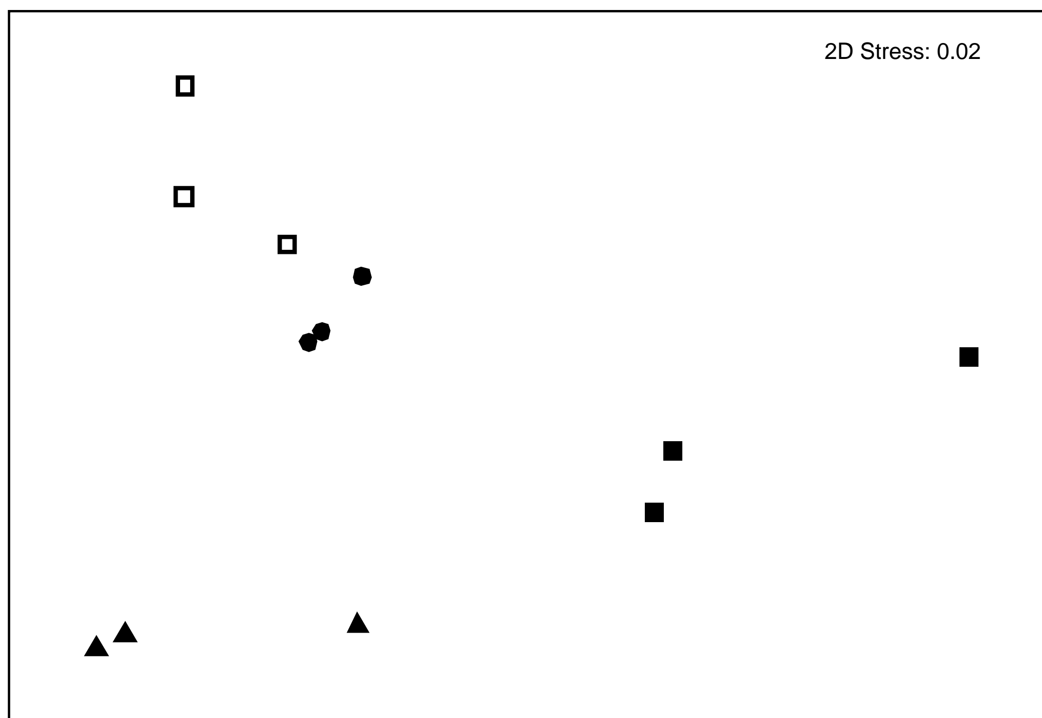


Table 3.5.3: ANOVA results for the top 20 terminal restriction fragments (TRFs) in the T-RFLP data, as ranked by abundance, for the effect of compaction and air infiltration on bacterial community structure.

TRF (bp)	Abundance rank	% Abundance	Cumulative % abundance	*P-values* Compaction	Air infiltration	Compaction*air infiltration
180	1	33.1	33.1	**<0.001** $^{-}$	0.202	0.903
77	2	17.3	50.4	0.104	0.336	0.299
76	3	9.0	59.4	0.328	0.592	0.827
115	4	8.7	68.0	0.126	0.264	0.592
499	5	5.0	73.0	0.013 $^{-}$	0.191	0.115
114	6	3.9	77.0	0.065	0.383	0.750
572	7	2.3	79.3	0.001 $^{-}$	0.179	0.179
495	8	2.2	81.5	0.113	0.113	0.113
488	9	1.8	83.3	0.168	0.168	0.168
221	10	1.8	85.1	0.004 $^{+}$	0.004 $^{-}$	0.004
220	11	1.8	86.9	0.033 $^{+}$	0.033 $^{+}$	0.033
155	12	1.7	88.6	0.003 $^{+}$	0.003 $^{-}$	0.003
575	13	1.7	90.3	0.203	0.203	0.041
492	14	1.5	91.7	0.128	0.128	0.128
301	15	0.9	92.6	0.082	0.082	0.082
160	16	0.9	93.5	0.200	0.200	0.200
517	17	0.9	94.4	0.089	0.089	0.089
519	18	0.7	95.1	0.002 $^{+}$	0.002 $^{+}$	0.002
476	19	0.7	95.8	0.083	0.083	0.083
601	20	0.6	96.4	0.081	0.081	0.081

Bold indicates a strong significant effect ($P < 0.001$); $^{+}$ = positive effect on TRF abundance, $^{-}$ = negative effect on TRF abundance

Experiment 3.6 Manipulating the ensilage of wilted, unchopped grass through the use of additive treatments

[J. McEniry, P. O'Kiely, N. J. W. Clipson, P. D. Forristal and E. M. Doyle]

This study aimed to quantify the effects of additive treatments with contrasting modes of action on the fermentation of wilted, unchopped herbage (as a model for baled silage), relative to that of precision-chopped herbage, under controlled conditions in laboratory silos. This information should help to identify additive treatments to be considered for use in baled silage production that would facilitate the creation of conditions that are more inhibitory to the activities of undesirable microorganisms and are more comparable to that of precision-chop silage. Previous studies have confirmed that unchopped and precision-chop grass ensiled in the laboratory silos used in the present experiment are suitable models for studying the ensilage of conventional baled and precision-chop silages, respectively.

Materials and methods: The experiment was organised in a 2 (chop treatments) x 6 (additive treatments) x 2 (stages of ensiling) factorial arrangement of treatments. Herbage was wilted for 24 h and representative samples were ensiled unchopped or precision-chopped in laboratory silos with the addition of one of six contrasting additive treatments. Each chop x additive treatment was replicated six times in a total of seventy-two laboratory silos. Half of these silos were opened and sampled after 2 days ensilage with the remaining silos opened after 110 days. Silage fermentation variables were assessed at both sampling times, while indices of nutritive value and silage aerobic stability were assessed after 110 days ensilage.

An homogenous plot of *Lolium perenne* (cv. Fennema) was mown (Pottinger, Nova 310T conditioner mower) on the 21 July 2004 and wilted in the field for 24 h with frequent tedding (Krone rotary tedder, KW550/4x7). There was no rainfall during wilting or harvesting.

After the wilting period, six representative herbage samples were taken prior to the chop treatment and ensiling for subsequent chemical and microbiological analyses.

The wilted herbage was then representatively sampled and used to fill 72 laboratory silos (height = 0.75 m, internal diameter = 0.152 m, internal volume = 13.6 l), and the herbage samples for thirty-six of these silos were precision-chopped (Pottinger, Mex VI) immediately prior to ensiling. The chopping knife number and feed roller speeds were chosen, according to the manufacturers instructions, to give a theoretical chop length of 19 mm. The remaining thirty-six silos were filled using samples of unchopped herbage.

Prior to filling the laboratory silos, six randomly selected samples (each 5 kg) of both unchopped and chopped herbage were assigned to each additive treatment. The following additive treatments (and application rates) were applied to the herbage: (1) no additive (control treatment), (2) lactic acid bacterial inoculant (LAB; Bio-Sil ®, *Lactobacillus plantarum* DSM 8862 and *Lb. plantarum* DSM 8866; Dr. Pieper technologie- und Produktentwicklung, GmbH), 1 g/t (3×10^5 colony forming units/g herbage), (3) sucrose, 5 kg/t, (4) lactic acid bacteria inoculant plus sucrose (both prepared and applied as above), (5) formic acid based additive (Add SafeR ®, 70 g ammonia and 640 g formic acid per kg additive; Trouw Nutrition, UK Ltd.), 3 l/t and (6) antimicrobial mixture (AMM; KofaSil ®, 80 g hexamethylene tetra-amine, 120 g sodium nitrite, 150 g sodium benzoate, 50 g sodium propionate and 600 g water per kg additive; Addcon Agrar, GmbH), 3 l/t. Each of the six treatments had 10 ml of liquid applied per kg of grass, which necessitated adding 0, 5, 7 or 10 ml distilled water per kg grass depending on the level of additive already applied. Aseptic techniques were used to prevent cross contamination between treatments.

A constant weight of 4 kg wilted herbage (additive applied was additional) was then ensiled, with the silos being packed manually and sealed immediately by a screw-on top with a rubber seal. Compaction was achieved in the silos by the inclusion of a 10.5 kg weight (diameter = 0.1 m) directly on the herbage to exert a continual vertical pressure (5.83 kPa). The laboratory silos were stored at a room temperature of 15 ºC prior to silo opening after 2 or 110 days ensilage.

Three silos from each treatment combination were sampled after 2 and 110 days of ensilage. Silage from each laboratory silo was weighed, and after thorough aseptic mixing, one sample per silo was taken.

All samples were stored at 4 °C prior to microbiological analyses (grass samples only) and at –18 °C for subsequent chemical analyses.

Results: Grass composition after the 24 h wilting period is as outlined in Table 3.6.1. For the main effect of chop, lactic acid concentration and the proportion of lactic acid in fermentation products were higher ($P < 0.001$), while butyric acid, ethanol and ammonia-N concentrations were lower ($P < 0.001$) in the precision-chop silage compared to the unchopped silage (Table 3.6.2). However, chopping had no effect ($P > 0.05$) on silage DM, pH, acetic acid, propionic acid or the total

concentration of fermentation products. Silage fresh weight loss, on average, was slightly higher ($P < 0.001$) for the unchopped compared to the chopped herbage (9 versus 7 g/kg) but the difference was small (Table 3.6.2).
Silage buffering capacity was higher ($P < 0.001$), while WSC concentration was lower ($P < 0.001$) in the precision-chop silage after 110 days ensilage (Table 3.6.3). Ash ($P < 0.05$), NDF ($P < 0.001$) and ADF ($P < 0.05$) concentrations were all slightly lower for the precision-chop silage (measured on day 110 only) but differences were modest in scale. Chopping had no effect ($P > 0.05$) on DMD or CP concentrations.
On average, the precision-chop silage was less stable on exposure to air after 110 days ensilage than the unchopped herbage (Table 4), with a shorter ($P < 0.001$) time to onset of heating, a higher ($P < 0.001$) maximum temperature rise and higher accumulated temperatures to 120 ($P < 0.01$) and 192 ($P < 0.001$) h.

For the main effect of additive, lactic acid concentration was highest ($P < 0.001$) for the LAB inoculant (Table 3.6.2). Correspondingly, the proportion of lactic acid in fermentation products was similarly lowest ($P < 0.05$) for the control (no additive) and sucrose treatments. On average, silage pH was lower ($P < 0.001$) for the LAB and the LAB + sucrose additive treatments. Ammonia-N concentration was lowest ($P < 0.001$) for the LAB + sucrose treatment and highest for the formic acid based additive. Ethanol ($P < 0.001$) and butyric acid ($P < 0.001$) concentrations were lowest for the AMM treatment, with the highest concentrations of these products observed in the control treatment. Propionic acid concentrations were low (< 1 g/kg DM) in all silages. Additive treatment had an effect ($P < 0.001$) on average silage fresh weight loss but the magnitude was relatively modest, with mean values for the main effect of additive treatment falling in the range of 6 – 10 g/kg, but with the AMM and formic acid treatments at the lower end of this range.
For variables measured on day 110, the main effect of additive indicated that the NDF concentration was slightly higher ($P < 0.01$) in the control treatment compared to other additives, while additive treatment had no effect ($P > 0.05$) on silage DMD, ash, CP and ADF concentrations (Table 3.6.3). Silage buffering capacity was similarly lower ($P < 0.001$) for the AMM and formic acid based additive treatments.
On average, the LAB + sucrose treated silage was the least stable on exposure to air after 110 days ensilage (Table 3.6.4), followed by the LAB only treatment. This was evidenced by a faster ($P < 0.001$) time to onset of heating, a higher ($P < 0.01$) maximum temperature rise and higher accumulated temperatures to 120 ($P < 0.01$) and 192 ($P < 0.001$) h.
Lactic acid, butyric acid, ethanol and ammonia-N concentrations were all higher ($P < 0.001$), while pH was lower ($P < 0.001$) after 110 compared to 2 days ensilage (Table 3.6.2). There was a greater decrease in pH from day 0 to day 2 of ensilage, than from day 2 to day 110. Acetic and propionic acid concentrations did not differ ($P > 0.05$) after 2 compared to 110 days of ensilage. Just over half of the concentration of ammonia-N on day 110 was present at day 2 (0.51), while 0.38 proportionately of lactic acid was present. The proportion of lactic acid in the fermentation products was higher ($P < 0.01$) after prolonged storage (110 d). Herbage fresh weight loss was higher ($P < 0.001$) after 110 days (12 versus 4 g/kg) than 2 days ensilage (Table 3.6.2).
When averaged across stage of ensiling, pH was lower ($P < 0.05$) for the control and sucrose additive treatments in the presence of chopping, while pH was higher ($P < 0.05$) in the chopped herbage after the addition of the AMM additive (Table 3.6.2). The lactic acid concentration was higher ($P < 0.001$) with chopping for all additive treatments, except for the LAB and LAB + sucrose treatments. The largest increase in lactic acid concentration due to chopping occurred for the control treatment.
Butyric acid concentration was low (< 1 g/kg DM) in all chopped herbages but an increase was observed due to the absence of chopping, with the concentration being highest ($P < 0.001$; 10 g/kg DM) in the control treatment, followed by the LAB and sucrose only additive treatments (~ 8 g/kg DM). Ammonia-N concentration was lower ($P < 0.01$) with chopping for each additive treatment, with the largest decrease in ammonia-N concentration due to chopping occurring for the control silage. There was no significant ($P > 0.05$) interaction between chop and additive treatment for DM, acetic acid, propionic acid, ethanol and fresh weight loss.
For the precision-chop silage after 110 days ensilage, the WSC concentration was similar (range 12 - 16 g/kg DM) irrespective of additive treatments (Table 3.6.3). However, in the unchopped silage, the WSC concentration was higher ($P < 0.01$) for the LAB and the LAB + sucrose treatments (31 and 38 g/kg DM, respectively). The interaction of chop and additive treatment was not significant ($P > 0.05$) for silage DMD, NDF, ADF, CP, ash or buffering capacity.

Precision chopping resulted in an increased ($P < 0.05$) accumulated temperature to 120 h for the control and the LAB treated silages, while having little effect on the aerobic stability of the other silages (Table 3.6.4). In the former silages and in the LAB + sucrose treated silage, chopping resulted in a large increase ($P < 0.01$) in accumulated temperature to 192 h.

When averaged across additive treatments, silage pH was lower ($P < 0.001$), while lactic acid ($P < 0.001$) and acetic acid ($P < 0.01$) concentrations were higher after 2 days ensilage in the unchopped compared to the chopped herbage (Table 3.6.2). The opposite was the case for each variable after 110 days ensilage.

Butyric acid ($P < 0.001$) was low (< 1 g/kg DM) for all treatments except for the unchopped silage after 110 days storage (11 g/kg DM). Ammonia-N concentration was similar on day 2 for both chop treatments but was higher ($P < 0.001$) in the unchopped herbage after 110 days storage. Silage fresh weight loss increased from day 2 to 110, but was higher ($P < 0.001$) for the unchopped compared to the precision-chopped herbage after 110 days ensilage. There was no significant ($P > 0.05$) interaction between chop and stage of ensiling for DM, propionic acid, ethanol and the proportion of lactic acid in fermentation products.

When averaged across chop treatments, silage pH and ammonia-N concentration were lowest ($P < 0.001$) for the LAB and the LAB + sucrose additive treatments after 2 days ensilage (Table 3.6.2). However, the silage pH was similar for all treatments at the end of the storage period (110 days). Ammonia-N concentration increased during ensiling for all treatments (from day 2 to day 110), but with the lowest ($P < 0.01$) concentration observed for the LAB + sucrose treatment after 110 days. Ammonia-N concentration was highest for the formic acid based treatment on day 2 and similarly higher for the formic acid based and control treatments after 110 days ensilage.

Butyric acid concentration was low in all silages at day 2, but increased during ensiling and was highest ($P < 0.001$) for the control treatment after 110 days ensilage. Butyric acid concentration after 110 days ensilage was lowest for the AMM additive, followed by the formic acid based treatment. The interaction of stage of ensiling and additive treatment had no effect ($P > 0.05$) on lactic acid, acetic acid, propionic acid or ethanol concentrations. Fresh weight loss was similar for all treatments on day 2 and increased during ensiling from day 2 to day 110. Fresh weight loss was lowest ($P < 0.01$) for AMM treatment after 110 days ensilage, followed by the LAB + sucrose and formic acid based additive treatments.

Butyric acid concentration was low (< 1.0 g/kg DM) in all silages after 2 days ensilage and low in the precision-chopped silage after 110 days storage (Table 3.6.2). However, the concentration increased ($P < 0.001$) in the unchopped herbage from day 2 to 110. Butyric acid concentration in the unchopped herbage after 110 days ensilage was < 5.0 g/kg DM for the AMM treatment only, while the concentration was > 10 g/kg DM for the control, sucrose and LAB additive treatments. The ammonia-N concentration was higher ($P < 0.05$) for each additive treatment on day 110 in the unchopped compared to the chopped herbage. The LAB + sucrose additive treatment inhibited the increase in ammonia-N concentration most in the unchopped herbage.

Herbage fresh weight loss was greater after 110 days ensilage for both chop treatments, but was higher ($P < 0.05$) for the unchopped compared to the chopped herbage (range 9 – 20 versus 8 – 11 g/kg; Table 3.6.2). There were no further significant interactions on any of the other variables measured.

Conclusions: The generally poorer fermentation in the unchopped compared to the chopped silage suggests that when similar grass is ensiled, at similar DM concentrations, as conventional unchopped baled or precision-chop silage, that there is a greater requirement for the fermentation to be assisted with the baled silage system. This could be achieved by more extensive wilting and/or by evenly applying adequate effective additives. Under such conditions, contrasting additives such as the LAB + sucrose, the formic acid based and the AMM additives could assist fermentation in baled silage, giving preservation approaching that of precision-chop silages.

A negative aspect from the use of the LAB based additives was the reduced aerobic stability of the resultant silage. This highlights the importance of maintaining adequate anaerobic conditions in the inoculant treated silage during storage. Poorer aerobic stability at feedout should not generally pose a major problem with baled silage in farm practice, as the bales would be consumed on most farms within 1 to 3 days of initial exposure to air. However, if assistance is required to improve aerobic stability, the AMM and the formic acid based additives were the most effective.

Further studies are required to establish whether similar responses for these additives can be obtained across a range of DM concentrations as with baled silage on Irish farms.

Table 3.6.1: The mean (s.d.) chemical (g/kg DM, unless otherwise stated, except for pH) and microbiological composition (log_{10} cfu/g herbage) of 24 h wilted herbage prior to ensiling

Dry matter (g/kg)	246 (3.4)
Dry matter digestibility (g/kg)	798 (11.8)
Ash	105 (3.4)
Crude protein	154 (4.4)
pH	6.06 (0.078)
Buffering capacity (m. Eq/kg DM)	471 (12.1)
Water soluble carbohydrate	170 (10.2)
Lactic acid bacteria	5.0 (0.49)
	4.5 (0.45)
	2.0 (0.35)
Bacilli	2.8 (0.25)
Yeast	2.6 (0.14)

Table 3.6.2: Treatment effects on silage pH, dry matter concentration, ammonia-N concentration, fermentation products and fresh weight loss (g/kg DM, unless otherwise stated, except for pH)

Treatment			DM	pH	LA	AA	PA	BA	Eth	L/ FP	NH_3-N	FWL
Chop	Additive	Stage of ensiling										
UC	Control	2	239	4.57	40	22	1.2	0.2	9.3	0.56	47	4
UC	LAB	2	244	4.33	72	15	0.3	0.2	8.5	0.75	38	4
UC	Sucrose	2	245	4.53	44	18	0.5	0.1	8.0	0.63	40	3
UC	LAB + Suc.	2	246	4.43	62	11	0.4	0.3	6.0	0.78	34	4
UC	Formic acid	2	252	4.70	34	11	0.4	0.3	5.0	0.68	55	2
UC	AMM	2	257	4.57	46	15	0.7	0.1	4.2	0.70	43	3
PC	Control	2	246	4.73	46	12	0.4	0.2	5.1	0.74	44	5
PC	LAB	2	249	4.56	50	10	0.3	0.1	4.9	0.77	38	8
PC	Sucrose	2	251	4.70	44	14	0.3	0.1	5.0	0.70	40	4
PC	LAB + Suc.	2	250	4.56	43	8	0.3	0.2	3.6	0.78	33	5
PC	Formic acid	2	249	4.80	34	9	0.4	0.2	2.9	0.74	66	2
PC	AMM	2	249	4.87	39	12	0.9	0.1	2.5	0.73	45	4
UC	Control	110	237	4.23	101	8	0.7	19.9	24.5	0.66	122	20
UC	LAB	110	247	4.13	113	8	0.5	14.7	19.8	0.73	97	17
UC	Sucrose	110	242	4.30	96	11	0.7	15.5	21.2	0.66	103	19
UC	LAB + Suc.	110	243	4.07	114	9	0.6	8.8	18.3	0.77	70	15
UC	Formic acid	110	247	4.30	102	12	0.7	5.4	12.8	0.77	102	13
UC	AMM	110	240	4.07	107	21	1.0	3.2	9.0	0.77	92	9
PC	Control	110	244	3.90	148	15	0.6	0.6	13.1	0.83	77	11
PC	LAB	110	244	3.90	136	14	0.4	0.7	14.8	0.82	62	10
PC	Sucrose	110	246	3.90	138	19	0.5	0.3	15.2	0.80	68	9
PC	LAB + Suc.	110	247	3.90	130	15	0.1	0.3	12.5	0.83	62	8
PC	Formic acid	110	246	3.90	116	15	0.4	0.7	10.9	0.81	88	10
PC	AMM	110	243	4.03	127	25	1.1	0.6	9.8	0.78	83	8
		s.e.m.[1]	3.8	0.046	5.2	4.0	0.24	1.28	2.32	0.047	4.2	1.1
Levels of significance												
Chop			NS	NS	***	NS	NS	***	***	***	***	***
Additive			NS	***	***	NS	*	***	***	*	***	***
Stage of ensiling			**	***	***	NS	NS	***	***	**	***	***
Chop x additive			NS	*	***	NS	NS	***	NS	NS	**	NS
Chop x stage of ensiling			NS	***	***	**	NS	***	NS	NS	***	***
Additive x stage of ensiling			NS	***	NS	NS	NS	***	NS	NS	**	**
Chop x additive x stage of ensiling[1]			NS	NS	NS	NS	NS	***	NS	NS	*	*

UC = unchopped, PC = precision-chopped; Control = no additive; LAB = lactic acid bacteria inoculant; LAB + Suc. = lactic acid bacteria inoculant + sucrose; AMM = antimicrobial mixture; DM = dry matter (g/kg), LA = lactic acid, AA = acetic acid, BA = butyric acid, PA = propionic acid, Eth = Ethanol, L/ FP = proportion of lactic acid in fermentation products (g/g), NH_3-N = ammonia-N (g/kg N), FWL = fresh weight loss (g/kg), NS = not significant, * = P < 0.05, ** = P < 0.01, *** = P < 0.001, [1] s.e.m. relates to 3 factor interaction

Table 3.6.3: Treatment effects on silage buffering capacity and on silage chemical composition indices (g/kg DM, unless otherwise stated) after 110 days ensilage.

Treatment		BC	DMD	NDF	ADF	Ash	CP	WSC
Chop	Additive							
UC	Control	892	760	496	296	113	160	18
UC	LAB	918	773	479	293	111	158	31
UC	Sucrose	878	771	485	284	113	155	15
UC	LAB + Sucrose	902	774	469	285	108	151	38
UC	Formic acid	830	786	465	289	107	155	21
UC	AMM	842	781	467	289	110	157	15
PC	Control	988	765	464	283	110	153	12
PC	LAB	978	776	463	283	109	155	13
PC	Sucrose	982	779	462	281	106	151	16
PC	LAB + Sucrose	928	776	457	278	107	154	16
PC	Formic acid	863	785	455	282	107	151	14
PC	AMM	891	769	456	280	108	156	11
	s.e.m.[1]	14.3	7.2	4.6	7.1	2.1	2.9	2.9
Levels of significance								
Chop		***	NS	***	*	*	NS	***
Additive		***	NS	**	NS	NS	NS	***
Chop x additive		NS	NS	NS	NS	NS	NS	**

UC = unchopped, PC = precision-chopped; Control = no additive; LAB = lactic acid bacteria inoculant; AMM = antimicrobial mixture; BC = buffering capacity (m. Eq/kg DM), DMD = dry matter digestibility (g/kg), NDF = neutral detergent fibre, ADF = acid detergent fibre, CP = crude protein, WSC = water soluble carbohydrates; NS = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, [1] s.e.m. relates to the interaction between chop and additive treatment

Table 3.6.4: Treatment effects on silage aerobic stability after 110 days ensilage.

Chop	Additive	Time to temp. rise > 2°C (h)	Maximum temperature rise (°C)	Time to maximum temperature (h)	Accumulated temp. rise to 120 h (°C)	Accumulated temp. rise to 192 h (°C)
UC	Control	192	0.6	118	1.4	2.2
UC	LAB	120	2.2	104	5.1	9.4
UC	Sucrose	192	0.9	12	2.7	4.3
UC	LAB + Sucrose	91	5.5	127	8.4	17.0
UC	Formic acid	166	1.5	137	3.1	6.5
UC	AMM	192	1.0	69	3.6	5.6
PC	Control	93	10.8	103	14.8	32.4
PC	LAB	45	10.9	66	23.2	61.6
PC	Sucrose	192	1.5	121	3.7	6.0
PC	LAB + Sucrose	38	17.6	53	3.1	69.4
PC	Formic acid	147	2.1	124	4.7	8.8
PC	AMM	192	0.9	127	3.1	4.7
	s.e.m.[1]	24.9	2.44	29.4	5.55	8.00
Levels of significance						
Chop		**	***	NS	**	***
Additive		***	**	NS	**	***
Chop x additive[$]		NS	NS	NS	*	**

UC = unchopped, PC = precision-chopped; Control = no additive; LAB = lactic acid bacteria inoculant; AMM = antimicrobial mixture; NS = not significant, * = P < 0.05, ** = P < 0.01, *** = P < 0.001, [1] s.e.m. relates to the interaction between chop and additive treatment

4. Optimisation of the plastic film surrounding wrapped bales of silage

Specifically, the aims of this part of the project were:

1. Identify the optimal types of plastic and sealing agents for wrapping bales
2. Identify the optimal configuration of plastic film to securely create adequately anaerobic conditions.

Experiment 4.1. Uni-axial stretching of baled silage wrap films: gas permeation properties

[Laffin, C., Mc Nally, G.M., Forristal, P. D., O'Kiely, P., and Small, C. M.]

This experiment investigated the effect of uni-axial stretching on the CO_2 permeation properties of LDPE/LLDPE films for use in baled silage application. The gas permeation properties of the film are affected by the crystallinity and molecular orientation of the films, and so differential scanning calorimetry (DSC) and polarized FTIR analysis techniques were used to determine the crystallinity and orientation (film stretching) of these films.

Materials and methods: The materials used in this work are shown in Table 4.1.1, and were manufactured from a LDPE-LLDPE blend (70/30: w/w). Film C produced from resin batch 5200 contained 8% polyisobutylene (PIB) to investigate the effect of tack additive on gas permeation. Films A and C from resin batch grade ML4518 and 5200 were chosen to investigate the effect of co-monomer type and had similar densities and melt flow indices (MFIs). SL4102 (Film B) was chosen to investigate the effect of density on gas permeation.

The films were manufactured using a Killion cast film extrusion system, which was fitted with a 600mm flexible lip sheet die, with a die gap set at 250µm. The barrel temperatures were increased from 200^oC at the feed section to 220^oC at the die. A constant screw speed of 30rpm was used; and the chill roll temperature was maintained at approximately 15^oC, and an air gap of 110mm. A rubber-coated roller was used to press the hot film extrudate onto the chill roll. 25µm film was obtained with a haul off ratio of 1.04 and nip roll speed of 7.0m/s.

Films were stretched from 0 to 300% at regular intervals using a specially designed clamping system on an Instron 4411 Universal Tensile Tester. A load cell of 0.1kN was used with a constant cross head speed of 250mm/min, and an initial gauge length of 125mm.The stretching apparatus consisted of a wide clamp (160mm) that was connected directly onto the Instron 4411. A film specimen, 160mm wide was mounted in the clamp and was then stretched to the desired level, at which point a frame was used to secure a section of the stretched film and ensure film tension for transfer to the Davenport permeation apparatus.

A Davenport gas permeability measuring apparatus was used to determine the permeation coefficient of film samples. The apparatus was designed for direct measurement of the gas transmission rate (GTR) through plastic films in accordance with B.S.2782, method 821A, ASTM D. 1434 and similar methods. The test gas used was CO_2 (99.8% purity), and 5 samples were tested for each film at each stretch level. Equation 1 was then used to determine the gas permeation coefficient.

Equation 1: $P = (273/76)(Vl/ATp_{up})(dp_{down}/dt)$

where; P = gas permeation coefficient ($cm^3(STP)cm^{-1}cm^2cmHg$), V = Volume of free space below polymer sample (20.33cm^3), A = Surface area of polymer sample (23.77cm^2), l = Thickness (cm) p_{up} = Pressure above specimen (76 cmHg), p_{down} = Pressure below specimen, and T = Temperature (298^oK).

The crystallinity of the various films was determined (Differential scanning calorimetry (DSC)) from the latent heat of fusion (H J/g) thermograms using a Perkin Elmer DSC-6. The samples were heated from 30 to 140^oC at a rate of 10 deg C/min. Spectra were obtained (Fourier transform infrared (FTIR) spectroscopy analysis) using a Perkin-Elmer FTIR spectrometer (spectrum 1000) fitted with a 0.12µm zinc selenide grid polariser. The equipment was positioned in a laboratory maintained at 25±1^oC. The instrument operated with a resolution of 1cm^{-1} and 40 scans were obtained for each sample. The IR absorbance scans were analysed between 700cm^{-1} and 750cm^{-1}, for changes in the intensity associated with the a- and b-axes.

Results: The film with the lowest density, Film B (0.903g/cm^3), exhibited the highest initial permeation coefficient; however this film also exhibited the greatest reduction in permeation coefficient upon stretch (Table 4.1.2). Films A and C (8%PIB) of similar density (0.918 and 0.917 g/cm^3), exhibited similar initial permeation coefficients throughout stretching. However, it was shown that at higher stretch values, above 140%, film C containing 8%PIB exhibited a greater reduction in permeation coefficient in comparison to film A. The permeation coefficient, the tensile force during stretch and the crystallinity for all films varied at different stretch levels. Film B exhibited a high tensile yield value with a large increase in deltaH occurring as a result of the initially low crystallinity and high chain entanglement in the film. The effect of chain branching on tie molecules increases the resistance to disentanglement, therefore when the yield point

was reached a large reduction in load was recorded. The increase in crystallinity and molecular orientation resulted in a decrease in the permeation coefficient of the film, thus improving the gas barrier properties. Film C exhibited an increase in crystallinity with increasing stretch due to stress-induced crystallisation. This increase in crystallinity and molecular orientation resulted in a reduction in the permeation coefficient of the film. Film A exhibited little change in overall crystallinity up to 100% stretch, with only a slight increase being recorded on further stretching beyond this point. A slight change in deltaH was also observed between 60 to 100% stretch, with first an increase and then decrease, that correlated to a secondary yield point. The secondary yield point was believed to be associated with irreversible lamellar fragmentation and reorganisation to a 'sheesh kebab' structure, resulting in higher molecular orientation and reduction in mechanical properties. It is proposed that chain slip occurs more easily in the thinner lamellae, by virtue of the smaller length of chain that has to slide through the lamellae, which would encourage the fine chain slip mechanism. Therefore, both lamellar thickness and overall crystallinity contribute to this double yielding effect. This high level of molecular orientation and increase in crystallinity leads to a decrease in the permeation coefficient of the film.

From previous analysis carried out on these films it was shown that each film exhibited different crystallinity, and thus crystallographic texture, leading to different crystallographic texture evolution upon deformation in the second regime explaining the differences in reduction of the permeation coefficients.

The increase in crystallinity for all stretched films could be attributed to stress-induced crystallisation due to straining of the amorphous component of the film, with the stress-induced crystals being smaller in size and therefore a lower thermodynamic stability resulting in a reduced melting point. This was observed for all films, with an increased peak found at 110°C with increasing stretch level.

A considerable decrease occured in the intensity of the 730cm^{-1} band with increasing stretch, and only a slight decrease occurred in intensity of both peaks at 720 and 730cm^{-1}, which is associated with a decrease in film thickness with increasing stretch.

All films exhibited an increase in orientation in the MD representing an increase of the b/a ratio. A decrease in the a-axis band also indicated greater intensity associated with the c-axis as a result of greater stress in this axis, and greater alignment with the incidence beam. The reduction in the a-axis band (730cm^{-1}) and an increase intensity associated with the c-axis therefore indicates that the lamellae tilt or slip with increase stretch during transition into a more fibrous morphology which occurs during cold drawing. This change was observed to occur at around 80-100% stretch indicated by little or no change being observed for the b/a ratio in the MD beyond this point.

Conclusions: This work investigated the effect of uni-axial stretching on the CO_2 permeation properties of films. It was shown that the CO_2 permeation coefficient of all films significantly decreased for the stretched films, with the largest reduction in permeation coefficient exhibited in the film of lowest density. All films were shown to increase in crystallinity and molecular orientation with increasing stretch, exhibiting a double yield point at around 80-100% stretch. Therefore, it can be concluded that the reduction in CO_2 permeation coefficient is as a result of the combination of an increase in the film crystallinity and molecular orientation.

Table 4.1.1. Technical specification of resins

Film	Manufacturer	Grade	Co-monomer	Density g/cm^3	MFI g/10min
A	Exxon	ML4518	Hexene	0.918	4.5
B	Dow	SL4102	Octene	0.903	1.5
C	Dow	5200	Octene	0.917	4.0
				Masterbatch 52 +/- 2%PIB in LLDPE	

Table 4.1.2. Effect of uni-axial stretching on the permeation coefficient, crystallinity and orientation of films
$^{+}$PC = CO_2 permeation coefficient ($cm^3(STP)cm^{-1}cm^2cm(10^{-6})$). PC SEM = 0.76, deltaH SEM = 0.75. Stretch (S), Polymer (P) and SxP were each significant at P<0.001 for PC and deltaH.

Polymer				Stretch								
Film	Density (g/cm^3)	MFI (g/10min)		1.0	1.2	1.4	1.6	1.8	2.0	2.4	2.7	3.0
			PC^{+}	37.8	31.6	26.2	22.8	19.1	20.4	19.6	16.6	10.9
			Force (mN)	0.0	32.8	36.7	38.7	39.4	38.6	38.4	38.8	38.9
A	0.918	4.5	ΔH	64.3	70.9	67.8	61.0	72.0	64.0	67.0	78.0	85.0
			b/a ratio MD	1.2	1.9	2.3	2.7	2.6	2.4	2.3	2.3	2.3
			b/a ratio TD	1.3	1.4	0.0	1.6	1.7	1.7	1.8	2.2	2.0
			PC^{+}	63.8	52.1	41.4	33.7	30.1	32.2	22.3	24.6	25.1
			Force (mN)	0.0	32.0	22.4	24.6	25.9	26.3	26.5	27.0	27.7
B	0.903	1.5	ΔH	16.3	39.8	33.6	37.1	42.8	36.1	39.6	49.7	51.1
			b/a ratio MD	1.2	1.6	1.8	2.1	2.5	2.8	2.6	2.7	2.7
			b/a ratio TD	1.3	1.3	1.2	1.2	1.3	1.7	1.6	1.8	2.0
			PC^{+}	34.6	25.3	25.9	24.0	22.1	17.9	22.2	13.0	
			Force (mN)	0.0	35.0	33.3	33.7	33.6	33.2	32.6	32.8	33.3
C	0.917	4.0	ΔH	51.0	61.8	56.4	56.1	59.6	59.9	67.2	67.0	60.2
			b/a ratio MD	1.3	1.7	2.3	2.7	2.8	2.5	2.9	2.9	2.7
			b/a ratio TD	1.4	1.3	1.2	1.4	1.4	1.5	1.7	1.8	1.8

Experiment 4.2. Carbon dioxide permeation properties of polyethylene films used to wrap baled silage

[Laffin, C., Mc Nally, G.M., Forristal, P. D., O'Kiely, P., and Small, C. M.]

Polythene stretch-film for wrapping baled silage must possess good gas barrier and mechanical properties to ensure satisfactory levels of anaerobosis are achieved and maintained in the bale. This experiment determined the CO_2 barrier properties of a number of commercially available films.

Materials and Methods Five commercial films (designated A to E) were investigated. Film C was reported to have been pre-stretched during manufacture. In each case a single layer of black film was tested, with 5 samples being obtained from the top, middle and bottom sections of a roll of film. A Davenport gas permeability measuring apparatus was used to determine the gas transmission rate (GTR) and permeation coefficient (PC) of film samples to CO_2 (99.8% purity). The GTR was measured according to B.S.2782, method 821A, ASTM D. 1434 and indicates the volume of gas transmission, with PC indicating the permeation per unit thickness Youngs modulus was measured from tensile analysis of the films in both the machine and transverse directions.The co-monomer type of the films was predicted from differential scanning calorimetry (DSC) analysis.

Results: Table 4.2.1 shows that the gas transmission rates (GTR) for films A, B, D and E were in good agreement with other published reports for 25µm polyethylene film. The GTR is influenced by film thickness, with Film C (thickness 12µm) showing the highest GTR ($49x10^3$ $ml/m^2/day/atm$). Film C also exhibited the lowest permeation coefficient ($24x10^{-6}$ml at standard temperature and pressure (STP)cm^2 versus 31 – $36x10^{-6}$ml(STP)cm^2). The higher GTR of film C at least partially reflected its lower thickness (12µm), but the lower permeation coefficient appeared to be as a result of pre-stretching during manufacture leading to greater crystallinity and molecular orientation, which would improve the barrier properties of the film. Mechanical analysis also showed significant variation (P<0.001) in the Youngs modulus across the width of the roll, with the largest variation being recorded for film D (108.3 – 146.5MPa in the machine direction). Film C exhibited the lowest Youngs modulus of all five films given its different co-monomer type. All films showed significant variation (P<0.001) in mechanical and gas permeation properties across the width of the roll, indicating non-perfect manufacture.

Conclusions: All films had similar permeation coefficient and Youngs modulus values, except for Film C which was pre-stretch and had a different co-monomer. The considerable variability in permeation and mechanical properties across the width of all rolls of film indicates an aspect of manufacture that needs improvement.

Table 4.2.1 CO_2 permeation and mechanical properties of five commercial films tested

				Location in roll of film (L)				Significance		
Film(F)	Co-monomer	Thickness (μm)		Top	Middle	Bottom	s.e.m[1]	Location	Film	L. x F.
A	Octene	25	GTR[#]	27.7	27.2	38.5	1.5	***	***	***
			PC[+]	27.7	28.6	37.6	1.1	***	***	***
			YMm[a]	103.6	125.0	116.6	2.5	***	***	***
			YMt[b]	129.3	135.5	140.5	4.0	***	***	***
B	Octene	25	GTR[#]	35.3	33.6	45.1	1.5	***	***	***
			PC[+]	32.5	33.0	42.9	1.1	***	***	***
			YMm[a]	133.2	117.7	131.8	2.5	***	***	***
			YMt[b]	151.2	150.7	132.5	4.0	***	***	***
C	Hexene	12	GTR[#]	49.8	47.4	49.1	1.5	***	***	***
			PC[+]	25.8	23.5	21.7	1.1	***	***	***
			YMm[a]	98.9	111.1	115.1	2.5	***	***	***
			YMt[b]	109.6	128.4	136.5	4.0	***	***	***
D	Octene	25	GTR[#]	34.3	37.6	41.3	1.5	***	***	***
			PC[+]	33.4	34.0	40.5	1.1	***	***	***
			YMm[a]	108.3	120.3	146.5	2.5	***	***	***
			YMt[b]	135.5	140.2	172.8	4.0	***	***	***
E	Octene	25	GTR[#]	34.9	31.6	34.5	1.5	***	***	***
			PC[+]	38.4	33.4	35.6	1.1	***	***	***
			YMm[a]	115.9	117.8	124.8	2.5	***	***	***
			YMt[b]	117.8	127.3	125.3	4.0	***	***	***

[#]GTR = CO_2 gas transmission rate ((ml/m^2/day/atm)(10^3)) [+]PC = CO_2 permeation coefficient (ml(STP)cm^2(10^{-6}))
[a]YMm = Youngs modulus machine direction (MPa) [b]YMt = Youngs modulus transverse direction (MPa) [1]s.e.m = L. x F.

Experiment 4.3. The effect of extrusion conditions and material properties on the gas permeation properties of LDPE/LLDPE silage wrap films

[Laffin, C., Mc Nally, G.M., Forristal, P. D., O'Kiely, P, and Small, C. M.]

This experiment investigated the effect of manufacturing conditions and material properties with varying concentrations of PIB on the gas permeation properties of such LDPE/LLDPE films for use in baled silage application.

Materials and methods: The materials used in this work are shown in Tables 4.3.1 and 4.3.2. Films manufactured from materials shown in Table 4.3.1 were processed as a blend due to previously reported problems associated with blown film extrusion of LLDPE, such as bubble instability and random bubble distortion. Therefore films were produced using a LDPE-LLDPE blend (70/30: w/w), containing 0, 2, 4, 6 and 8% PIB Masterbatch. Films manufactured from materials shown in Table 4.3.2 were produced by the cast film extrusion process, and again were manufactured using a LDPELLDPE blend (70/30: w/w), for a direct comparison, containing 0 and 8% PIB masterbatch (Table 4.3.1).

The blown films were manufactured using a Killion-KN150 38mm Extruder, using a general purpose screw (L/D 30, 3:1 compression ratio), connected to a 75mm diameter annular die with a die gap of 800 μm. The temperature profile of the barrel was increased from 195°C at the feed section to 210°C at the die. A single orifice cooling ring was used to cool the melt upon exit from the die. The screw speed was set at 20rpm, with the haul-off adjusted to produce films of 25μm thickness. Films were manufactured at blow-up ratios (BUR) of 1.5, 2.0 and 2.5. Cast films were manufactured using a Killion cast film extrusion system, which was fitted with a 600mm flexible lip sheet die, with a die gap set at 250μm. The barrel temperature was increased from 200°C at the feed section to 220°C at the die. A constant screw speed of 30rpm was used; with a maintained chill roll temperature of approximately 15°C, with an air gap of 110mm. A rubber-coated roller was used to press the hot film extrudate onto the chill roll. 25μm film was obtained with a haul off ratio of 1.04 and nip roll speed of 7.0m/s.

A Davenport gas permeability measuring apparatus was used to determine the gas transmission rate (GTR)(permeability) of film samples. The apparatus was designed for direct measurement of the GTR through plastic films in accordance with B.S.2782, method 821A, ASTM D. 1434 and similar methods. The test gas used was CO_2 (99.8% purity). Equations 1 and 2 were then used to determine the gas transmission rate and gas permeation coefficient respectively.

Equation 1: $GTR = ((273pV)(24x10^4))/ATP$

where; p = rate of pressure change (cm Hg/hr), V = Total volume of space (20.33 cm³), A = Area of specimen (23.77 cm²), T = Temperature (298°K), and P = Pressure difference (76 cmHg).

Equation 2: $P = (273/76)(Vl/ATp_{up})(dp_{down}/dt)$

where; P = gas permeation coefficient (cm³(STP)cm⁻¹cm²cmHg), V = Volume (20.33cm³), A = Area (23.77cm²), l = Thickness (cm), p_{up} *= Pressure above specimen (76 cmHg),* p_{down} *= Pressure below specimen, and T = Temperature (298°K).*

Differential Scanning Calorimetry (DSC) analysis was carried out using a Perkin Elmer DSC-6, with samples heated from 30°C to 140°C at a rate of 10 deg C/min. The latent heat (ΔH J/g) was calculated for each sample. Shrinkage analysis was carried out in accordance with ASTM D2732. A circular test sample, of diameter 50mm, was cut and its orientation with respect to the manufacturing process marked. It was then lightly coated with silicone oil and placed on the hot plate, at 130 deg. Celsius for 75 seconds, after which it was measured and the shrinkage determined.

Results: Film thickness details are shown in Tables 4.3.3 and 4.3.4. There was no significant correlation between PIB content and change in CO_2 transmission rate and permeation coefficient for these films (Fig.4.3.1 and Fig 4.3.3). This is particularly noticeable for films manufactured by cast extrusion. It is also evident that the manufacturing conditions do have an affect on the permeation properties of the films since changes in manufacturing conditions may cause changes in crystallinity and orientation of the films. The films A – D, manufactured from materials of the same density but different MFI, had similar permeation properties indicating that the MFI of the material does not affect the permeation properties of the film (Fig 4.3.2 and Fig 4.3.4) . However, it is clear from films G and I, films with lower densities, that the density of the material has a significant affect on the permeation property of the film.

For films A - D manufactured from hexane copolymers, the results show that the gas transmission rate and permeation coefficient is reduced by the presence of 8% PIB Masterbatch. The results also show a similar trend for films manufactured from low density material, films G and I. Films E, F and H, manufactured from the octene based resins, show no relationship between PIB content and permeation coefficient. The gas

transmission rates for all films tested showed good agreement with previous reports for 25µm polyethylene film.

Films manufactured by cast extrusion process exhibited lower crystallinity than those films manufactured by blown film extrusion, as a result of improved quenching of the film during manufacture due to good contact between the melt and the chill rolls. Crystallinity of the cast films also shows a slight increase with increasing PIB content.

Films manufactured using blown film extrusion showed little correlation with respect to blow up ratio and crystallinity, which is reflected in the CO_2 permeation properties of the films.

Ingredient density had an affect on the crystallinity of the films produced - films with higher crystallinity exhibited improved gas barrier properties, and films A, B, D, G and I showed increased crystallinity with the presence of PIB and thus improved gas barrier properties. The orientation of these films has been previously investigated showing that films manufactured from octene co-polymers showed a higher degree of orientation in the machine direction than the transverse direction, while films manufactured from hexene and butane copolymers exhibited similar orientation in both directions. Therefore these results show that co-polymer type and PIB content have an affect on the crystallization of the film, which is further altered by the presence of PIB in hexene and butene co-polymer films.

From shrinkage analysis on all films it was shown that the greatest shrinkage was exhibited in the machine direction. Films manufactured from cast extrusion exhibit less shrinkage in comparison to films manufactured from blown film extrusion in both directions. For films containing 0 and 8% PIB it was shown that as the blow up ratio increases that the shrinkage of the films increases. For films manufactured from varying manufacturing conditions it was shown that the PIB content does not appear to have an affect on the shrinkage characteristics of the film. Films manufactured from octene co-polymers exhibit a greater difference in shrinkage characteristics between machine and transverse directions indicating a greater degree of orientation in the machine direction. The addition of PIB reduced the degree of orientation of the films, and this was more noticeable in films manufactured from hexane copolymers.

Conclusions: This work investigated the effect of processing conditions and resin properties and PIB content on the CO_2 permeation properties of films. Permeation analysis of the various films showed that extrusion processing conditions affected the CO_2 permeation properties of the films. These changes in barrier performance may be attributed to changes in crystallinity, shown in Figure 5, and orientation induced by processing which has been previously reported. The results also show that the MFI of the material had little or no effect on the CO_2 permeation properties of the various films. Polymer density was shown to have the most significant effect on barrier performance, with materials of low density having significantly poorer gas barrier properties than materials of a higher density. For films manufactured with octene co-polymer the results show that the concentration of PIB had no significant effect on the gas barrier properties of these films. However, films manufactured from hexene and butene copolymers showed a decrease in CO_2 permeation coefficient and increase crystallinity with increasing concentration of PIB masterbatch. DSC analysis showed that materials with higher densities were more crystalline, and exhibited improved gas barrier properties. Shrinkage analysis (130°C) showed that films manufactured from octene copolymers exhibited a greater degree of orientation, and incorporation of PIB (8%) reduced orientation of the films, and this was particularly noticeable for films manufactured from hexene copolymers. The greater degree of orientation of octene films was also shown to reduce the gas permeation coefficient.

Table 4.3.1. Material properties used in manufacture of films

Material type	Producer	Density g/cm^3	MFI g/10min
LLDPE	Dow	0.919	1.1
LDPE	Dow	0.924	0.8
PIB	Polytechs	0.912	200-300
	Masterbatch 52+/- 2%PIB in LLDPE		

Table 4.3.2. Differential material properties used in manufacture of films

Notation	Manuf.	Grade	Co-monomer	Density g/cm^3	MFI g/10min
A	Exxon	ML4518	Hexene	0.918	4.5
B	Exxon	ML2518	Hexene	0.918	2.5
C	BASF	18 RFA	Hexene	0.918	2.5
D	BASF	18 FAX	Hexene	0.918	1.1
E	Dow	5200	Octene	0.917	4.0
F	Dow(ZN)	NG5056E	Octene	0.919	1.1
G	Dow	SL4102	Octene	0.903	1.5
H	Dow	5100	Octene	0.920	0.85
I	BASF	0332H	Butene	0.905	1.0

Table 4.3.3. Thickness of films manufactured from varying processing conditions

Thickness (micron)	0%PIB	2%PIB	4%PIB	6%PIB	8%PIB
BUR 1.5	25.8	27.4	29.8	27.4	29.6
BUR 2.0	22.6	24.0	30.6	24.3	26.4
BUR 2.5	22.4	29.6	29.2	30.4	31.6
CAST	21.2	23.8	25.8	25.4	21.4

Table 4.3.4. Thickness of films manufactured from different materials

Thickness (micron)	A	B	C	D	E	F	G	H	I
0%	26.2	21.6	22.0	18.4	21.8	21.2	20.4	20.0	19.6
8%	24.0		21.4	19.4	24.0	21.4	21.4	22.0	18.4

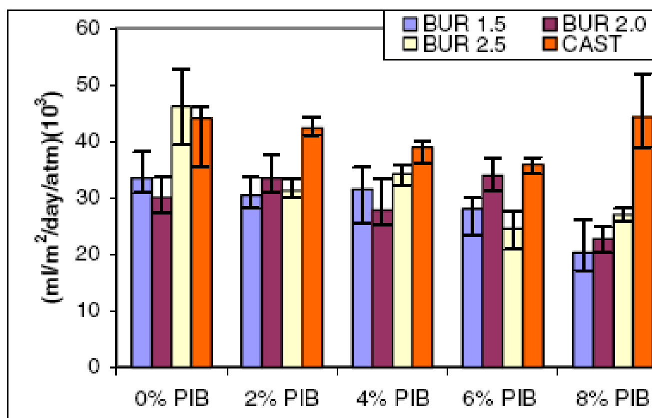


Fig 4.3.1: The effect of BUR and PIB on gas transmission rate

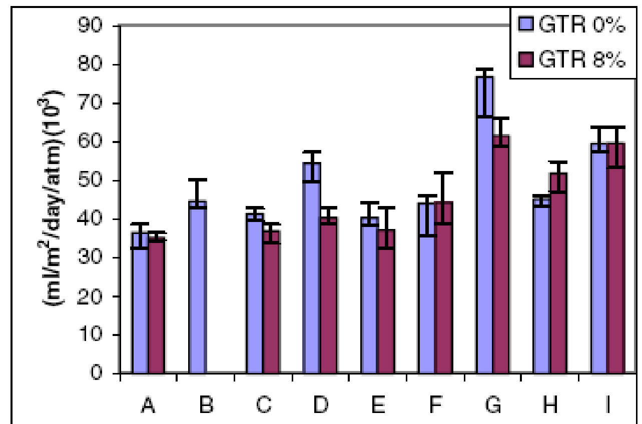


Fig 4.3.2: The effect of material properties and PIB on gas transmission rates

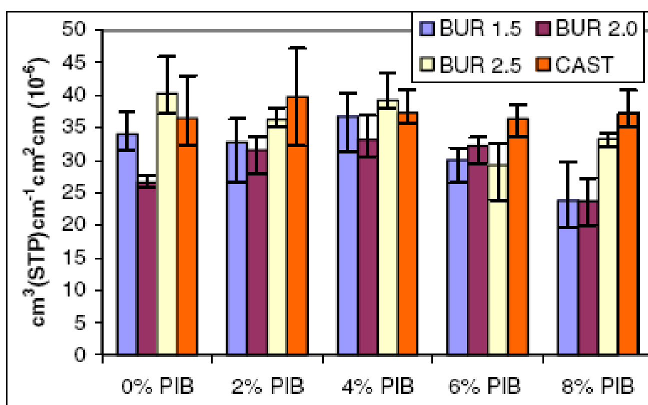


Fig 4.3.3: The effect of BUR and PIB on permeation coefficient

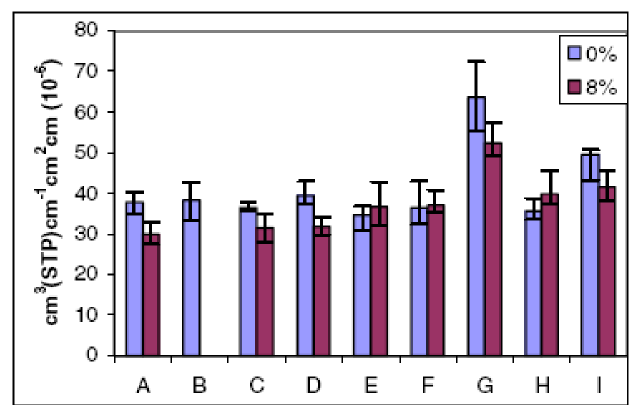


Fig 4.3.4: The effect of material properties and PIB on permeation coefficient

Experiment 4.4. Characteristics of four different types of PVC resins by the blown film extrusion technique

[Laffin, C., Mc Nally, G.M., Forristal, P. D., O'Kiely, P., and Small, C. M.]

In this experiment films were manufactured from four different types of PVC resins by the blown film extrusion technique, with the various properties investigated. Carbon dioxide permeation analysis was carried out to determine the gas barrier properties of the films, while mechanical analysis of the films was conducted to determine the mechanical properties of the films for comparison to polyethylene films.

Materials and methods: The technical specifications of the various PVC resins used are shown in Table 4.4.1. The polyvinyl chloride blown films were manufactured using a Killion-KN150 38mm Extruder, with a general purpose screw (L/D 30, 3:1 compression ratio), connected to a 75mm diameter annular die with a die gap of 800µm. The temperature profile of the barrel was increased from 155°C at the feed section to 170°C at the die. A single orifice cooling ring was used to cool the melt upon exit from the die. The screw speed was set at 10rpm, with the haul-off adjusted to produce films of 85µm thickness. Films were manufactured at one blow up ratio of 1.5.

Tensile analysis of these PVC films was conducted in accordance with ASTM D882-95 using an Instron 4411 Universal Tensile Tester. Ten replicates were performed per sample. The CO_2 permeation properties of the PVC films were determined using a Davenport gas permeation cell, using the manometric method, with five replicates performed per sample . The thermal properties of the films were determined using differential scanning calorimetry (DSC). Analysis were conducted using a Perkin Elmer DSC-6 with samples heated from 40^{0}C to 150^{0}C at a rate of 10^{0}C /min. The films manufactured from the 4 types of PVC resin were uni-axially stretched to obtain a comparison to the LLPE films previously investigated in this report. The CO_2 permeation properties of the films were obtained at regular intervals from 0 to 200% of their original length. The films were stretched at 250mm/min using the set of specially designed clamps attached to the Instron 4411 Universal Tensile tester.

Results: *Mechanical properties:* Films manufactured from the PVC2 resin exhibited the lowest break strength (11.96 MPa) in the MD of the four films investigated, with films manufactured from the PVC3 showing the lowest break strength (8.3 MPa) in the TD. However, films manufactured from the PVC3 resin exhibited the greatest break strength (28.14 MPa) in the MD, with films manufactured from PVC1 resin exhibiting the greatest break strength (25.26 MPa) in the TD. The results also show that all films, with the exception of those manufactured from the PVC1 resin, exhibited greater break strength in the MD in comparison to the TD.

These results also show that the break strength of the PVC films are comparable to the commercially available polyethylene.

Films manufactured from the PVC2 resin exhibited the greatest elongation at break in both the MD and TD directions for all the films investigated. The lowest elongation at break in the MD was recorded for films manufactured from the PVC1 resin (319.66 %), while films manufactured from the PVC3 resin exhibited the smallest elongation at break (42.98 %) in the TD. However, the elongation at break of the PVC films (~450 %) was considerably lower than that recorded for the commercial polyethylene films (~700 %).

The Young's Moduli values of the PVC films analysed show variation in value depending on the resin type used during manufacture. From the analysis it was shown that films manufactured from the PVC2 resins exhibited the lowest moduli in both the MD and TD of all the films, while films manufactured using the PVC1 exhibited the greatest moduli in the MD and TD of the films analysed. The modulus results also show comparable results were obtained for films manufactured from the PVC1 and PVC3 films. From these results it was shown that the modulus of the film was dependant on the resin density, with films manufactured from the lowest density resin (PVC2 with a density of 1.16 g/cm^3), exhibiting the lowest modulus values, and films manufactured from higher densities exhibiting greater modulus values (PVC1 and PVC3 with density of 1.25 g/cm^3). Comparison to modulus values recorded for commercial polyethylene films also shows significantly higher values for the polyethylenes in comparison to those obtained for films manufactured from PVC resins.

Tear analysis was carried out on the films using the Instron 4411 Universal Tensile Tester in accordance with to ASTM D1938-94. Ten replicates were performed per sample.

These results show that all films exhibited greater tear resistance in the transverse direction (TD), with the exception of films manufactured from the PVC2 resin that showed similar tear strengths in both the machine direction (MD) and TD. The greater tear resistance in the TD would indicate a

greater degree of molecular orientation in the MD. It was also shown that films manufactured from the PVC2 resin exhibited lower tear strength, in both directions, in comparison to the other films analysed indicating that the resin density is an important factor contributing to the ultimate tear strength of the film. Comparison of the tear analysis of the PVC films with films manufactured from LDPE/LLDPE resins showed that the tear strength of the PVC films (~0.4 MPa) was considerably lower to that obtained for the polyethylene films (~4 MPa).

Diffusion and permeability Films manufactured from the PVC 2 resin exhibited the greatest diffusion coefficient, while films manufactured from the PVC1 and PVC 3 resins, of same density ($1.25 g/cm^3$), exhibited similar diffusion coefficients. The lowest diffusion coefficient was observed for the PVC 4 films. Comparison to polyethylene films shows that much greater diffusion coefficients are obtained for PVC films (~0.3 versus ~6 $cm^2/sec\ 10^{-6}$). These results would indicate that the gas molecules move more easily through the PVC films, due to a lower tortuosity factor as a result of the non-presence of crystallites. Films manufactured from the PVC 2 resin (lowest density), exhibited a high solubility coefficient, however, similar values were recorded for films manufactured from the PVC 4 resin. Films manufactured from the PVC 1 and PVC 3 resins exhibited similar coefficients, which were considerably lower in comparison to the other films.

Films manufactured from the PVC 2 resins exhibited the greatest permeation coefficient (200.9 $cc/m^2/day$), and, therefore, worst barrier properties, of all the films analysed. The other films exhibited similar permeation properties, with films manufactured from the PVC 1 resin exhibiting the lowest coefficient value (41.8 $cc/m^2/day$). From the results of the permeation analysis it can be said that the main factor affecting the permeation properties of the film is the density of the resin used in manufacture, which is a similar trend to that obtained for polyethylene films as shown in previous sections of the report, however, the solubility coefficient is the primary factor determining the barrier property in PVCs compared to crystallinity in polyethylenes. The resin information shows that the PVC 2 resin has a K value of 75 in comparison to a value of 70 for the other films. This would suggest that the PVC 2 possess a greater amount of plasticiser. The addition of plasticisers to a polymer results in an increased segmental mobility and usually in an increased penetrant transport.

Thermal properties: The DSC thermograms showed that small endotherms were recorded at temperatures around 58°C, which is associated with the Tg of PVC resins. However, no further endotherms were recorded beyond this point indicating that all resins were non crystalline due to the effects of plasticiser.

The diffusion coefficient of the films analysed exhibited little or no change with uni-axial stretching, with the exception of films manufactured from the PVC 2 resin which exhibited a significant increase in diffusion with increasing stretch to 200%. An increase in solubility with increasing stretch for films manufactured from the PVC 4 resin, and a decrease in solubility with increasing stretch was recorded for films manufactured from the PVC 2 resin. For films manufactured PVC 1 and PVC 3 resins, some changes were observed with increasing stretch, however, no clear trend was recorded.

Stretching effect: The effect uni-axial stretch, on the CO_2 permeation coefficient of the various PVC resins, indicated that for all films manufactured from PVC resins, no change in the CO2 permeation coefficient was recorded with increase in uni-axial stretching from 0 to 200% of the original film length, with the exception of films manufactured from the PVC 2 resin which showed some increase with increased stretching. At this stage little literature exists on the CO_2 permeation properties of PVC films. In the case of films manufactured from the PVC 2 resins, the stretching leads to a greater percentage of plasticiser being exposed to the surface. It is known that the addition of plasticiser increases the segmental mobility of the polymer. As a greater percentage of plasticiser is exposed during stretching the segmental mobility of the polymer increases resulting in an increase in diffusion coefficient and a decrease in solubility coefficient. For all the other films analysed, it can be said that no change is observed as a result of pPVC being an amorphous polymer, and therefore no increase in crystallinity is recorded, and thus the tortuosity of the film is not altered. Comparison of the stretch analysis results obtained with those for polyethylene films showed that the CO_2 permeation coefficient values from 0 to 200% stretch were greater for the PVC films, with films manufactured using the PVC 2 resin exhibiting the greatest coefficient values.

Conclusions: Films were manufactured from four different PVC resin types. Tensile analysis of the films showed that the break strength of the films were comparable to those of polyethylene films. However, it was shown that the elongation at break and Young's moduli obtained were lower than those obtained for polyethylene films, with the elongation and moduli being dependant

on the resin density. Tear analysis of the films showed that all films, with the exception of those manufactured from the PVC 2 resin, exhibited greater tear resistance in the TD indicating a greater degree of molecular orientation in the MD. As with the results obtained for tensile analysis, the tear strength of the films was shown to be dependant on the resin density. Comparison of tear analysis of the PVC films with results obtained for polyethylene films again showed considerably lower values for the PVC films.
Permeation analysis of the un-stretched films showed that the greatest CO_2 permeation coefficient was obtained for films manufactured from PVC 2 resins, with all other films exhibiting similar permeation properties. These results indicated that the primary variable in determining the barrier property of the film was the density and plasticiser content of the resin used during manufacture. The uni-axial stretch analysis of the films showed that all films exhibited no change in CO_2 permeation coefficient with increase in uni-axial stretching, with the exception of films manufactured from PVC 2 resins that exhibited some increase with uni-axial stretching as a result of increased plasticiser exposure. Comparison of these results to films manufactured from polyethylene films showed that films manufactured from PVC resins exhibited greater CO_2 permeation coefficients with increased uni-axial stretching.
Overall, it can be concluded that comparison of films manufactured from PVC resins to those manufactured from polyethylene resins, the PVC films demonstrated inferior properties to those desired in films intended for use in silage wrap applications.

Table 4.4.1. Properties of polymers used in manufacture of films for investigation of PVC resin type

Material type	Producer	Grade	Density g/cm^3	K value
PVC 1	Colorite	95-226B-04	1.25	70
PVC 2	Colorite	5011-02	1.16	75
PVC 3	Colorite	95-226B-02	1.25	70
PVC 4	Colorite	7511B-00	1.22	70

5. Other

Experiment 5.1. Quantify the interactions between harvesting system and forage dry matter content, and within baled silage between the number of layers of plastic stretch-film and the duration of storage, in terms of conservation characteristics.

[O'Kiely, P., Forristal, P.D. and McEniry, J.]

Recent experiments using laboratory silos indicated that the main factors likely to influence the preservation of baled silage are anaerobiosis >>> forage DM content >> compaction > chopping. Thus, wrapping bales properly with adequate effective plastic stretch-film is vital, with the penalty for imperfect sealing likely to be greater for forage of higher DM content. The effects of this interaction are likely to increase as the duration of storage of wrapped bales increases. One surprising outcome from Experiments 3.2 and 3.3 was that the fermentation of long and chopped forage were not as different as hypothesised – the reasons for this are not evident. Thus, this experiment compared baled and precision-chop forage harvested at different DM contents and, within baled silage, quantified the effects of forage DM content, anaerobiosis (number of layers of wrap), the duration of bale storage and their interactions.

Material and methods. Grass was harvested at three DM contents (stages of wilting) using a precision-chop harvester or a round baler, and these were ensiled for 6 months. Forage harvested with the baler was wrapped with 4 (standard in Ireland), 6 (standard in Sweden) or 8 (secure for long-term storage of dry herbage) layers of film, and 'opened' after 6 or 18 months storage. Some bales were stored with only 2 layers of stretch-film (6 months). Forage was weighed and samples chemically analysed before and after ensilage, and the extent of mould growth quantified. The treatments were:

1. Grass harvested at 200gDM/kg using a precision-chop harvester; open after 6 months
2. Grass harvested at 350gDM/kg using a precision-chop harvester; open after 6 months
3. Grass harvested at 500gDM/kg using a precision-chop harvester; open after 6 months
4. Grass harvested at 200gDM/kg using a round baler; wrap in 2 layers stretch-film; open after 6 months
5. Grass harvested at 200gDM/kg using a round baler; wrap in 4 layers stretch-film; open after 6 months
6. Grass harvested at 200gDM/kg using a round baler; wrap in 6 layers stretch-film; open after 6 months
7. Grass harvested at 200gDM/kg using a round baler; wrap in 8 layers stretch-film; open after 6 months
8. Grass harvested at 200gDM/kg using a round baler; wrap in 4 layers stretch-film; open after 18 months
9. Grass harvested at 200gDM/kg using a round baler; wrap in 6 layers stretch-film; open after 18 months
10. Grass harvested at 200gDM/kg using a round baler; wrap in 8 layers stretch-film; open after 18 months
11. Grass harvested at 350gDM/kg using a round baler; wrap in 2 layers stretch-film; open after 6 months
12. Grass harvested at 350gDM/kg using a round baler; wrap in 4 layers stretch-film; open after 6 months
13. Grass harvested at 350gDM/kg using a round baler; wrap in 6 layers stretch-film; open after 6 months
14. Grass harvested at 350gDM/kg using a round baler; wrap in 8 layers stretch-film; open after 6 months
15. Grass harvested at 350gDM/kg using a round baler; wrap in 4 layers stretch-film; open after 18 months
16. Grass harvested at 350gDM/kg using a round baler; wrap in 6 layers stretch-film; open after 18 months
17. Grass harvested at 350gDM/kg using a round baler; wrap in 8 layers stretch-film; open after 18 months
18. Grass harvested at 500gDM/kg using a round baler; wrap in 2 layers stretch-film; open after 6 months
19. Grass harvested at 500gDM/kg using a round baler; wrap in 4 layers stretch-film; open after 6 months

20. Grass harvested at 500gDM/kg using a round baler; wrap in 6 layers stretch-film; open after 6 months
21. Grass harvested at 500gDM/kg using a round baler; wrap in 8 layers stretch-film; open after 6 months
22. Grass harvested at 500gDM/kg using a round baler; wrap in 4 layers stretch-film; open after 18 months
23. Grass harvested at 500gDM/kg using a round baler; wrap in 6 layers stretch-film; open after 18 months
24. Grass harvested at 500gDM/kg using a round baler; wrap in 8 layers stretch-film; open after 18 months

There were six bales or clamps per treatment.

The analysis and interpretation of the data from this experiment was not complete in time for this report.

Experiment 5.2. Sampling procedures with baled silage

[P.D. Forristal, and P. O'Kiely]

The procurement of representative samples is a prerequisite for satisfactory experimentation. With conventionally stored bulk feeds, simple spatial sampling patterns within the stored feed are chosen to make it likely that samples are representative. Differences caused by storage height are usually accounted for by coring through, or sampling from, the full depth of an horizon. Baled silage presents a greater sampling challenge. Cylindrical 1.2 m x 1.2 m bales have approximately 50% of the silage volume in the outer 0.13 m periphery of the bale. The large surface area may also differentially influence silage preservation or sample characteristics at the periphery of the bale because of local temperature fluctuations and gas exchange. The aim of this experiment was to determine the variation in composition within bales, thereby allowing sampling methods to be evaluated.

Materials and Methods: Grass from the second harvest of a predominantly perennial ryegrass sward was baled after 1.5 days wilting in good conditions, using a fixed-chamber baler (1.2 m x 1.2 m). Eight bales were moved to a storage area where they were wrapped with 4 layers of 0.025 mm stretch film. After 240 days storage, the bales were subjected to the following sampling procedure. Core samples were taken at two depths (0 to 13 cm and 13 to 30 cm) from four distinct positions around the bale circumference. The sample at each position was a composite of 6 sub-samples taken equidistant across the 1.2 m wide convex curvature of the bale. The four positions were across the top of the bale (B) and parallel to this at 90^0 (C), 180^0 (D) and 270^0 (A) around the curvature. In total, 64 cored samples were produced (2 depths x 4 positions x 8 bales). Following core sampling (5 cm diameter, powered corer), each of the eight bales was completely chopped using a precision-chop forage harvester, and duplicate samples taken for each bale. Nutritive and ensiling characteristics of all samples were analysed.

Results: At ensiling, the grass had the following characteristics: 341 (s.d. 19.8) g dry matter (DM)/kg, 111 (s.d. 3.8) g crude protein (CP)/kg DM, 749 (s.d. 7.3) g digestible DM/kg and 95 (s.d. 2.7) g ash/kg DM. Silage fermentation was restricted due to the low water activity associated with the relatively high DM concentration. It was characterised by a high lactate to acetate ratio, but volatile fatty acid concentrations were in the order acetate < propionate < butyrate, despite ammonia-N concentrations being below 50 g/kg N. There was no interaction between depth of sampling and sampling position. Shallow sampling gave lower ($P<0.05$) DMD, WSC and pH values, but higher ($P<0.05$) ash, crude protein and acetic acid concentrations (Table 5.2.1). However, differences were small. The lower digestibility and WSC values at the bale surface suggest that localised gas or temperature conditions facilitated some sugar catabolism. There was no significant effect of sampling position on any of the measured variables.

Differences between core sampling and full bale chopped sampling were small (Table 5.2.2). Core sampling gave a lower DMD and pH and a higher ammonia-N than precision chopped samples.

Conclusion: In the conditions of this trial, most differences between the sampling methods assessed were quantitatively small. However, sampling depth was important, indicating that the method used must accurately represent the depth/volume profile of the bale. Current core sampling practices may give too much weight to the inner portion of the bale. Poorly sealed bales would be likely to have much greater differences between shallow and deep layers. While sample position had no effect in this trial, this result would not be expected with bales of lower DM concentration.

Table 5.2.1. Depth of core sampling and its influence on silage chemical composition

	Depth of sampling		SEM	Sign.
	Shallow (0-13cm)	Deep (13-30cm)		
Dry matter[1]	325	316	5.6	ns
DMD[1]	711	727	3.0	***
Ash[2]	101	97	0.9	*
C. protein[2]	113	108	1.2	**
pH	4.76	4.87	0.014	***
Ammonia-N[3]	39	39	1.4	ns
Lactic acid[2]	26	24	0.9	ns
Acetic acid[2]	3.7	2.8	0.23	**
Propionic acid[2]	7.1	6.2	0.34	ns
Butyric acid[2]	16	15	0.8	ns
Ethanol[2]	15	14	0.6	ns
WSC[2]	53	71	3.3	***

[1](g/kg), [2](g/kg DM), [3](g/kg N)

Table 5.2.2. Composition of core and precision-chop samples

	Core sampling	Precision-chop	SEM	Sign.
Dry matter[1]	320	329	4.8	ns
DMD[1]	719	732	3.6	*
Ash[2]	99	100	0.8	ns
C. protein[2]	111	110	1.4	ns
pH	4.81	4.89	0.011	**
Ammonia-N[3]	39	35	1.2	*
Lactic acid[2]	25	24	0.8	ns
Acetic acid[2]	3	3	0.3	ns
Propionic acid[2]	7	5	0.3	*
Butyric acid[2]	16	14	0.7	ns
Ethanol[2]	15	15	0.7	ns
WSC[2]	62	66	2.8	ns

[1](g/kg), [2](g/kg DM), [3](g/kg N)

Experiment 5.3. A gas monitoring facility for baled silage

[P.D. Forristal, D. Keppel and P.O'Kiely]

In Ireland, approximately one-third of the silage harvested is conserved in individually wrapped round bales. Satisfactory forage preservation with the system is dependent on the effectiveness of the polythene cover as a barrier to gas movement. Many factors influence the performance of the polythene, including the type and quantity of film used and how it is applied. The use of conventional silage analysis parameters to evaluate the performance of the polythene is not entirely satisfactory, as aerobic spoilage is usually restricted to the surface of the bale, making sampling and quantitative analysis difficult. Earlier work has shown the potential of bale gas analysis (particularly CO_2 contents) to indicate the effectiveness of the polythene cover. The infrequent sampling technique used in these trials did not give a complete picture of bale gas profiles over an ensiling period. The objective of the work described here was to construct a continuous gas monitoring facility for silage bales and to examine the gas profile over a storage period under controlled conditions.

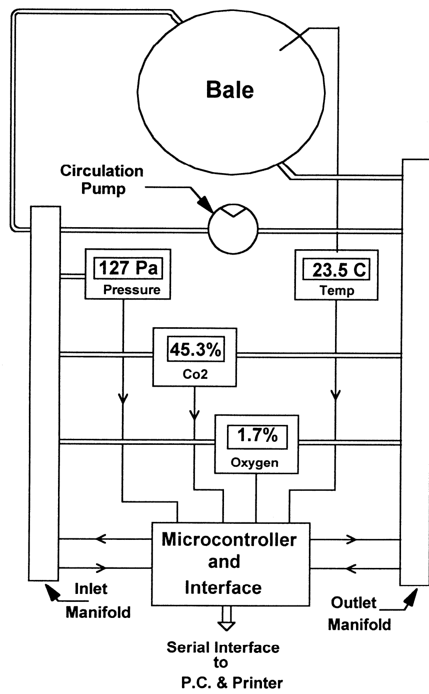
Materials and methods: An automatic gas sampling facility was designed and constructed. The main components of the system are represented schematically in Fig. 5.3.1. Four bale parameters are measured automatically: bale temperature; static gaseous pressure; bale gas CO_2 content; and bale gas O_2 content. A platinum resistance thermometer sensor inserted 100 mm into the bale surface is used to measure bale temperature. Static gaseous pressure is measured using a +1000 Pa

differential pressure sensor. An infra-red CO_2 analyser with a 0-100% range is used to monitor CO_2, while a paramagnetic oxygen sensor is used to assess O_2 levels. The electronic sampling/analysis unit selects the bale to be analysed, presents the gas to the sensors for analysis, and records all readings. Each bale is connected to an electronically controlled manifold by two gas pipes. At the start of the analysis cycle, the electronic controller opens two solenoid valves connecting the first bale to the manifold. The static pressure of the gas within the bale is read at this point. A pump then circulates gas within the bale for 2 minutes. After circulation, the gas is then passed through the O_2 and CO_2 sensors. Finally, the temperature of the bale is sensed. At each sampling time, all the sensed values are recorded onto a memory chip. The gas sampling system is a closed loop system where all of the sampled gas is returned to the bale. The sampling analysis and recording functions are controlled by a programmable electronic controller. Calibration of the gas sensors is effected by introducing calibration gases into the manifold.

A commissioning trial was put in place to check the functioning of the system and to collect bale gas analysis data. Eight bales of a 2nd-harvest, predominantly ryegrass sward, were wrapped with 4 layers of 25 micron thickness stretch-film. Four bales were placed inside a controlled environment facility (20°C), with the remainder stored in ambient conditions. The bales were wrapped and connected to the analysis system in less than 2 hours. At hourly intervals, CO_2, O_2, temperature and gaseous pressure values were recorded from all 8 bales. A separate data acquisition system was used to record air temperature and radiation close to the bales stored in ambient conditions. All data was recorded for a 240-day period.

Results: The automatic gas sampling system performed satisfactorily over the duration of the trial. The gas sensors functioned reliably, with relatively little drift between routine calibrations. However, the pressure sensor was less reliable, giving occasional erroneous readings. Data recording and subsequent downloading via laptop computer was successful. A summary of some of the measured parameters is presented in Figs. 5.3.2 and 5.3.3. The CO_2 and gaseous pressure profiles recorded over the first 6 days of the trial are presented in Fig. 5.3.2. Each line represents the mean of the bales in the trial. Recorded O_2 levels were less than 1% within 2 hours of wrapping. The level of CO_2 reached its maximum at approximately 6 days. The CO_2 profiles for the entire storage period are illustrated in Fig. 5.3.3. The marked degradation in CO_2 content over time confirms the trends obtained in previous trials where infrequent sampling was used.

Conclusion: A gas monitoring system was designed, constructed and tested. The sampling and recording functions performed satisfactorily, with expected outputs from the O_2 and CO_2 sensors. The internal pressure monitoring technique used may require further validation. The facility will allow the gaseous environment within bales to be monitored and should facilitate studies of polythene film wrapping configuration.



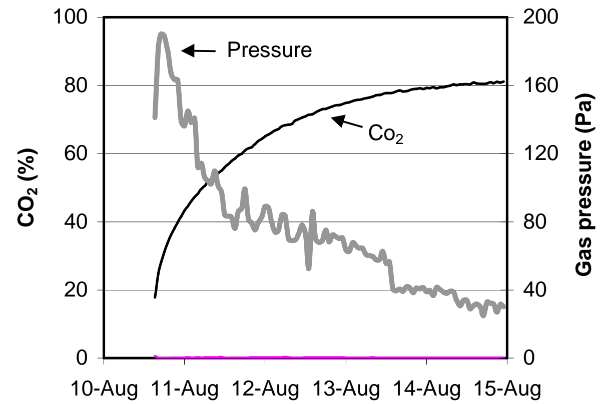


Fig. 5.2.2. CO_2 and gas pressure profiles of bales: Days 1-6.

Fig. 5.2.1. Schematic of the bale monitoring system

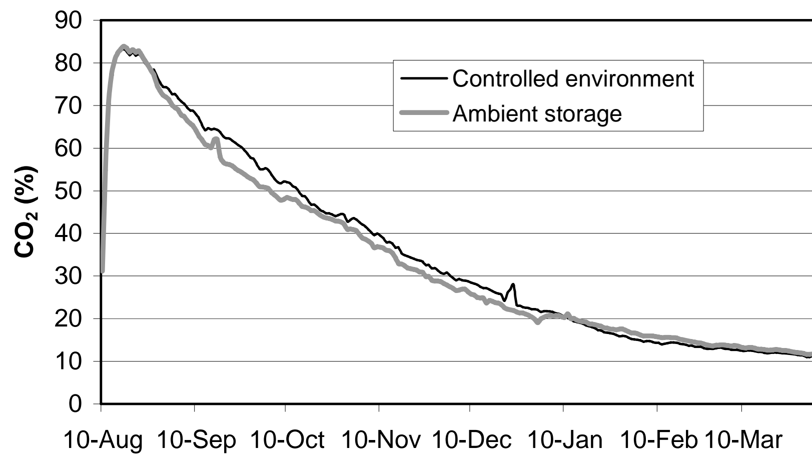


Fig. 5.2.3. CO_2 profiles of bales stored in ambient and controlled conditions for 140 days.

6. Conclusions

- Methodologies for sampling baled grass silage were developed to accurately reflect the relationship between depth and volume in the bale. Detailed sampling methodologies for fungal propagules and for visible mould or yeast colonies were developed.
- A substantial fungal spora can be found in baled silage and in order to prevent such propagules germinating and growing a high standard of bale wrapping, handling and storage is required. Once this high standard that ensures maintenance of an anaerobic environment within bales is achieved then fungal growth on the bales will be very restricted. The guidelines (Recommendations) for achieving this are presented in the next section of this report.
  Thus, good management can dramatically reduce the numbers and types of yeast and mould propagules in baled grass silage. Bales produced using normal on-farm procedures had higher mould counts (primarily of *P. roqueforti)* most likely because the integrity of the polythene film surrounding these bales was compromised during the storage period. When mould colonies were visibly present on baled silage, other parts of the bales were likely to have propagule counts that were raised.
- In a pilot survey, approximately 40% of the bales examined on farms had visible damage to the plastic film – this is likely an underestimation of the extent of damage to the film as damage that was not evident upon visual inspection was also likely. Bales with damaged film had more fungal coverage than where the film appeared intact.
- In a surveys of farms in the midlands and nationally, visible fungal growth occurred on 90% of bales. *P. roqueforti* had an almost ubiquitous distribution in bale collections, whereas *S. commune,* and to a lesser extent *P. fermentans,* was strongly influenced by the DM content of silage. A less extensive wilt may make bales less susceptible to *S. commune,* but will do little to prevent growth of *P. roqueforti* which is of more concern because of its ability to produce potentially harmful mycotoxins in silage. As air ingress *via* damaged film surrounding bales is a major factor permitting fungal growth, it is essential for livestock owners to ensure that the polythene film applied to bales does not become damaged. The absence of damage promotes a more extensive LAB fermentation while at the same time limits the supply of oxygen that would favour the growth of spoilage fungi.
- The secondary metabolites produced *in vitro* by *P. roqueforti* and *P. paneum* isolated from baled grass silage in Ireland were identified. Secondary metabolites produced by *P. roqueforti* and also presumably *P. paneum* were detected in visually mouldy silage contaminated with *P. roqueforti* and in silage samples that did not appear to be mouldy.
- The morphological, cultural and molecular characteristics of *P. roqueforti* and *P. paneum* isolated from baled grass silage in Ireland were recorded.
- The bacterial composition of typical baled and precision-chop grass silages were defined, with the former having higher counts of yeast and Clostridia, lower counts of Bacilli, and similar counts of lactic acid bacteria and Enterobacteria. Within bales, differences in chemical or microbiological composition between the outer 20 cm and the inner 40 cm were small.
- The onset of fermentation and the decline in pH were slower in baled compared to precision-chop silages. This permits the continuation of plant enzyme activity (especially in wetter herbages) and the persistence of greater numbers of Enterobacteria in the early stages of ensilage, both of which could impede achieving a successful preservation. The latter effect is further accentuated in baled silage, where wilting is an integral part of production, by the restrictive impact of wilting on fermentation. Furthermore, these effects and the numbers of undesirable microorganisms would be greater for farm silages where storage conditions can sometimes be less than ideal. The combined effects of factors such as the extent of wilting, harvester type (e.g. differ in the extent of physical disruption caused to herbage) and the effectiveness of excluding oxygen combine to create conditions within bales that are less conducive to inhibiting the activity of undesirable microorganisms than occurs in precision-chop silage. Thus, technologies are needed to improve the fermentation in baled silage.
- In general, laboratory silos were used with unchopped herbage as a relatively useful model for contrasting baled silage and precision-chop silage.

- The range of herbage DM concentrations and the impact of air ingress had a much greater effect on silage conservation characteristics than the compaction or chopping treatments studied. The main interactions were between the extents of wilting and air infiltration, wilting and compaction, and compaction and air infiltration. Air infiltration, as allowed in this trial, had a greater effect on in-silo losses than any of the other factors studied.
  A surprising outcome with many of the conservation variables studied was the absence of significant interactions between most of the factors. In many cases this appeared to be related to the absence of pore space effects perhaps limiting the extent of air infiltration through the silage. Pore space was not greatly affected by either chopping or compaction and consequently these factors had little effect on air ingress.
  The overwhelming objective with the baled silage system in particular, must therefore be to rapidly achieve adequately anaerobic conditions and maintain them thereafter. Failure to achieve this will lead to progressively greater losses, especially with drier herbage. The impacts of forage chopping or compaction are relatively minor if anaerobic conditions prevail. However, the evidence from Irish farm silages is that the technologies employed to achieve and maintain anaerobic conditions may be less successful with baled compared to conventional silages.
- Where good quality silages were produced under controlled conditions and little difference in silage quality and microbial composition were observed between ensilage systems, T-RFLP proved a potentially useful tool to study the ensilage process and for following the dynamics of bacterial community changes over the course of the fermentation. It generally agreed with findings from conventional analyses. In such a copiotrophic environment, where a restricted group of microorganisms are responsible for community changes, it is possible that culture-based approaches are adequate tools to study the changes in microbial composition. However, the use of culture-independent approaches could potentially result in the analysis of a greater extent of the community (e.g. uncultured *Lactobacillus sp*. were detected by cloning). Allied to this the rapid nature of some molecular methods, the range of methods available and the ability to target different microbial groups using specific primers shows the great potential of DNA-based methods in the analysis of complex microbial systems. Furthermore, a significant benefit of the T-RFLP method is that an overview of the whole bacterial population can be obtained in one assay. As a result, T-RFLP offers a new method to study the ensilage process and offers a viable alternative to culture-dependent methods.
- Both conventional microbiological and molecular data analyses showed similar shifts in bacterial community composition when contrasting ensiling treatments were compared. This change in community composition and the associated negative impacts on silage fermentation could be more pronounced in drier herbages where the fermentation would be more restricted and where air could be readily penetrate the silage mass.
- The chemical properties of polyethylene films and extrusion settings during manufacture can affect the characteristics of the film and consequently it's performance as a barrier to gas movement. In particular, monomer type, the quantity of PIB added and the blow-up ratio used in film extrusion can affect it's gas barrier properties. Pre-stretching films during manufacture can improve the barrier properties resulting in less gas transmission per unit of film thickness, but this can be at the expense of reduced physical performance.
- There is scope for further development work in the area of film stretching during application. The CO_2 permeation coefficient of all plastic films tested significantly decreased i.e. improved barrier performance, when the film was stretched, with the largest reduction in permeation coefficient exhibited in the film of lowest density. The increase in crystallinity and molecular orientation with increasing stretch contributed to this permeation change.
- The concept of using non-polyethylene films or film layer components may prove useful as preliminary work on alternative resins such as Nylon, PVC and oriented polypropylene indicated that these materials transmit less gas than polyethylene. However further studies on PVC indicated difficulties in achieving good barrier properties in all situations. More work on the polymers used for baled silage stretch film production to produce films with improved gas barrier and physical performance would be beneficial.
- The feasibility of continuously monitoring bale gas content and gaseous pressure within bales was illustrated allowing the effect of treatments such as the number of layers of film cover etc on these parameters to be assessed.

7. Recommendations

The experiments documented in this series of three reports on consecutive research projects on baled silage have led to the following set of recommendations for baled (1.2m cylindrical shaped) silage on Irish farms:

(a) Plan

Baled silage is now quite expensive to make, so it is important that the money a farmer invests in producing a meadow and in then mowing, baling, wrapping and storing it are adequately repaid. The chances of success are much higher where the major issues can be pre-planned rather than where making baled silage becomes a rushed or salvage operation. Thus, it is important to work out in advance

- how much baled silage is required and which fields it should come from
- approximately when it should be harvested
- how (or by whom) will it be mown, baled/wrapped and transported
- where it will be stored
- where and when it will be fed.

Wilting is an important component of making baled silage, so some flexibility is needed in the plan to ensure the meadow can be mown when weather conditions permit good field drying.

Baled silage in Ireland differs from conventional clamp silage in that most of the costs – baling, wrapping and transport - are incurred on a per bale (effectively per tonne) rather than a per hectare basis. Consequently there is a lesser penalty for conserving high-quality, low-yielding, grass crops for baled rather than conventional silage. This feature can be exploited on farms, and encourages conserving good quality grass, where the superior feeding value of a highly digestible bale more easily justifies its ensiling cost.

Finally, always incorporate the need for on-farm safety in all aspects of making, storing and feeding bales.

(b) Mowing

- manage mowing to promote effective wilting
- mow a meadow after the dew has evaporated. This is because a heavy dew on a high-yielding meadow contributes over three tonnes water per hectare and surface water usually dries quicker from a standing crop
- monitor weather forecasts, and try to mow and wilt during a spell of dry, sunny weather.

(c) Wilting

The radiant heat from direct sunshine together with the dryness of the air are what evaporate water from a wilting crop. Thus, the more of a mown meadow that is exposed to sunshine and air movement the better.

- the target is to bale the grass at about 300 to 350 g dry matter (DM)/kg herbage
- aim that the mown swath is on the ground for preferably one, and at most two, nights
- the grass treatment required to achieve this will depend on the yield of the crop and drying conditions - heavy crops in moderate drying conditions will require the most aggressive approach
- if a mower-conditioner is used, ensure that the gates behind the conditioner are opened so as to leave the grass in a wide swath. Aim for 60 to 80% ground cover.
- if available, use of spreader-mowers, which give up to 100% ground cover, can eliminate the need for separate tedding in certain conditions
- if it is planned to ted grass, do it as soon as feasible after mowing so as to maximise the duration that most grass is exposed to sunshine. If there is a risk of rain overnight, or if baling is expected to be carried out early the following morning, it may be advisable to windrow the tedded grass before nightfall.
- minimise the production of lumpy swaths.
- manage wilting with the aim of helping to subsequently facilitate making firm, regular-shaped bales

(d) Baling

Aim to make dense, well-shaped bales. These bales will be heavy. However, the reduced number of bales per hectare decreases baling, wrapping and transport costs. Well-shaped bales are easier to wrap properly and to handle without causing damage. They are also better at retaining their cylindrical shape during storage.

- aim to present well-shaped swaths that suit the baler pick-up
- adjust the baler density setting to the maximum allowable position
- drive the tractor to provide an even and balanced flow of grass into the bale chamber

- use a slow rather than a fast forward tractor speed, to help produce dense, well-packed bales
- balers with fixed knife choppers (i.e. ‘slicers’) can fit an additional 8-15% more grass into a bale, depending on factors such as the number and sharpness of knives and grass type. Thus a bale that would have weighed 500 kg should weigh 540 to 575 kg if slicing knives were fitted and are sharpened regularly.
- additives should not be required where effective wilting and appropriate sealing are achieved

(e) Wrapping

The simple but vital aim is to create oxygen-free conditions for the wrapped bale, and these conditions need to last through to feedout. When wrapped in 4 layers of conventional polythene (plastic) film, the thickness of such plastic on a silage bale is approximately 70 μm compared to 250 μm for a double-sheeted silage clamp. Since each bale of silage typically has 6 to 8 times the surface area in contact with plastic compared to clamp silage, it is clearly important that the plastic film is of good quality, is properly applied and is not subsequently damaged.

Bale wrappers are geared to apply film so that each layer overlaps the underlying layer by 50%. In Ireland, four layers of film are typically used, although in countries such as Sweden six layers are the norm. The number of revolutions of the wrapper turntable needed to ensure a nominal covering by 4 layers of film (750 mm) on a regular, well-shaped bale is at least 16 turns. In general, a minimum of 4 layers of conventionally applied standard stretch film is required to conserve baled silage adequately. Where good management practice prevails, the benefits accruing from progressing to 6 layers of film are small. However, where bales may be stored for a prolonged duration, 6 layers may be advisable. The production of dense, high dry matter (DM) bales from highly digestible grass, with in excess of 200 kg DM per bale, can justify the use of 6 layers of film. An increase of 25 kg DM per bale will pay for two extra layers of film by reducing the number of bales to be baled, wrapped, transported and stored.

- ideally transport bales to the final storage area and wrap them there. This avoids damage to the plastic wrap in the field caused by birds and reduces the risk of mechanical damage to the plastic during transport.
- count the number of wrapper turntable revolutions to ensure enough plastic film is applied
- ensure bale-drop table is working effectively to prevent damage as the newly wrapped bale is dropped onto the ground
- plastic film should be purchased from reputable sources only, and wrapping should be done in accordance with the wrapper and plastic manufacturers recommendations.
- ensure that the correct stretch is being used (generally 70% for conventional film) - some new thin films require a lesser stretch

(f) Handle/transport

Gentle handling of bales before and after wrapping is essential in order to maintain the shape of the bales and the integrity of the seal provided by the plastic film.

- avoid rough handling of unwrapped bales as this can mis-shape them, thereby rendering them more difficult to wrap perfectly (avoid using a spike to move bales prior to wrapping)
- transport wrapped bales to the storage area immediately after wrapping, and certainly within 24 hours. An extended delay can allow the shape of some bales to settle, making them more difficult to mechanically lift without causing damage to the plastic film.
- examine bale handling equipment for damage/rough areas (e.g. remove rust or file down rough steel) , and ensure that the operator uses the bale handler with great care
- immediately repair any damage to the plastic film
- considerable amount of damage to the plastic film caused by birds can take place in the field shortly after wrapping. If wrapped bales cannot be transported immediately to the storage area, relocating them temporarily from the silage stubble to an area of green grass will usually avoid bird damage.

(g) Storage

- select an uncluttered storage site from which the bales can be conveniently and safely removed at feedout time
- chose a level, smooth and hard surface on which to store the bales
- if the bales were made from wet grass, store the wrapped bales on a concrete pad from which effluent can be securely collected
- bales stored at ground level can be positioned on their curved or flat sides

- successfully wilted bales can be stored up to 3 tiers high if suitable bale handling equipment is available (max of two high if in REPS). Once stored two or more tiers high, the bales should be on their curved side.
- unwilted bales should be stored at ground level

(h) Protect and inspect

- fence off the stored bales from livestock, etc.
- ideally, stacks of wrapped bales should be protected at the sides and top with fine mesh netting or monofilament lines (0.5 m spacing). Aim to have about a 0.5 m gap between the netting and the bales.
- whereas painted markings on bales do not provide fully secure protection from bird damage, they can significantly reduce the scale of damage. Use white emulsion paint (not oil based) on black plastic. 'Eye' shaped designs are more effective than X or O designs.
- cats are a frequent cause of damage to stored bales, but it is not so easy to prevent access by them to the bales
- take appropriate steps to reduce the risk of rodents
- inspect the bales regularly for any damage to the plastic film, and repair damage immediately

(i) Feeding

- bales can be fed to cattle at any stage after wrapping
- aim to have bales consumed within two days of unwrapping
- physically remove and discard mouldy bales or parts of bales and do not offer to livestock. Wear an appropriate face-mask, gloves, etc., when handling mouldy forage, and adopt appropriate hygiene precautions afterwards.

(j) Collect plastic

- collect the plastic wrap after removal from bales, and dispose of it in an environmentally benign manner

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Go raibh míle maith agaibh!!

9 References

O'Kiely, P., Forristal, P.D., Brady, K., McNamara, K., Lenehan, J.J., Fuller, H. and Whelan, J. (2002). Improved technologies for baled silage. Beef Production Series # 50 (End-of-project report for RMIS 4621), 128 pages.

O'Kiely, P., Forristal, D. and Lenehan, J.J. (1999). Baled silage. Beef Production Series #11, Teagasc, Grange Research Centre, 44 pages.

10. Publications (to date)

Brady, K.C., O'Kiely, P., Forristal, P.D. and Fuller, H. (2005). Schizophyllum commune on big-bale grass silage in Ireland. Mycologist, 19 (1): 30-35.

McEniry, J., O'Kiely, P, Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2006). The microbiological and chemical composition of baled and precision-chop silages on a sample of farms in County Meath. Irish Journal of Agricultural and Food Research, 45: 73-83.

McEniry, J., O'Kiely, P, Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2007). The relative impacts of wilting, chopping, compaction and air infiltration on the conservation characteristics of ensiled grass. Grass and Forage Science, 62 : 470-484.

McEniry, J., O'Kiely, P, Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2007). The microbiological and chemical composition of silage over the course of fermentation in round bales relative to that of precision-chop silage. Animal Feed Science and Technology (in review).

McEniry, J., O'Kiely, P, Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2007). Bacterial community dynamics during the ensilage of wilted grass. Journal of Applied Microbiology (in review)

McEniry, J., O'Kiely, P, Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2007). Manipulating the ensilage of wilted, unchopped grass through the use of additive treatments. Irish Journal of Agricultural and Food Research (in press).

McEniry, J., O'Kiely, P, Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2008). Assessing the impacts of various ensilage factors on fermentation in grass silage using trad. culture and bacterial community analysis techniques (in preparation).

McNally, G.M., Laffin, C., Forristal, P.D. , O'Kiely, P. and Small, C.M. (2005). The effect of extrusion conditions and material properties on the gas peremeation properties of LDPE/LLDPE silage wrap films. Journal of Plastic Film and Sheeting, 21: 27-38.

O'Brien, M., Nielsen, K.F., O'Kiely, P., Forristal, P.D., Fuller, H.T. and Frisvad, J.C. (2006). Mycotoxins and other secondary metabolites produced *in vitro* by *Penicillium paneum* Frisvad and *Penicillium roqueforti* Thom isolated from baled grass silage in Ireland. Journal of Agricultural and Food Chemistry, 54: 9268-9276.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H.T. (2005). Fungi isolated from contaminated baled grass silage on farms in the Irish midlands. FEMS Microbiology Letters, 247(2): 131-135.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H.T. (2006). A note on sampling baled grass silage for fungal propagules. Journal of Animal and Feed Sciences, 15: 305-311.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H.T. (2007). Quantification and identification of fungal propagules in well-managed baled grass silage and in normal on-farm produced bales. Animal Feed Science and Technology, 132: 283-297.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H.T. (2007). Visible fungal growth on baled grass silage during the winter feeding season in Ireland and silage characteristics associated with the occurrence of fungi. Animal Feed Science and Technology, (in press).

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H.T. (2007). Fungal contamination of big-bale grass silage on Irish farms: predominant mould and yeast species and features of bales and silage. Grass and Forage Science (in review).

O'Brien, M., Egan, D., O'Kiely, P., Forristal, P.D., Doohan, F.M. and Fuller, H.T. (2007). Morphological and molecular characterisation of *Penicillium roqueforti* and *Penicillium paneum* isolated from baled grass silage. Mycological Research (in review).

Forristal, P.D. and O'Kiely, P. (2005). Update on technologies for producing and feeding silage. Silage production and utilisation. Proceedings of the 14th International Silage Conference (and a satellite workshop of the 20th International Grassland Congress) (eds. R.S. Park and M.D. Stronge), Belfast, 3 to 6 July 2005. Wageningen Academic Publishers, p83-96.

Forristal, P.D. and O'Kiely, P. (2004). Baled silage: an overview of its development and stretch wrapping needs. Proceedings of the Third Polymer Processing Research Symposium, Queens University Belfast, 28-29 January, 10 pages.

Forristal, P.D., Keppel, D. and O'Kiely, P. (2003). A gas monitoring facility for baled silage. In: Proceeding XIII International Silage Conference, Auchincruive, Scotland, 11-Sep-2002, p 420-421.

Forristal, P.D. and O'Kiely, P. (2003). Sampling procedure with baled silage. In: Agricultural Research Forum, Tullamore, 03-Mar-2003, ISBN 18417 4016, p 116 8106
Laffin, C., McNally, G.M., Forristal, P.D. , O'Kiely, P. and Small, C.M. (2004). The effect of extrusion conditions and material properties on the gas peremeation properties of LDPE/LLDPE silage wrap films. Proceedings of the ANTEC Conference, Chicago, Illinois, USA, 16-20 May, 2004. Volume 1: 230-234.
Laffin, C., McNally, G.M., Forristal, P.D. and O'Kiely, P. (2004). The effects of extrusion conditions and material properties on the gas permeation properties of LDPE/LLDPE silage wrap films. Proceedings of the Third Polymer Processing Research Symposium (ed. G.M. McNally), Queens University Belfast, 28-29 January, 21-29.
Laffin, C., McNally, G.M., Forristal, P.D., O'Kiely, P. and Small, C.M. (2005). Carbon dioxide permeation properties of polyethylene films used to wrap baled silage. Proceedings of the 14th International Silage Conference (and a satellite workshop of the 20th International Grassland Congress) (eds. R.S. Park and M.D. Stronge), Belfast, 3 to 6 July 2005. Wageningen Academic Publishers, p251.
Laffin, C., McNally, G.M., Forristal, P.D., O'Kiely, P. and Small, C.M. (2005). Uni-axially stretching LDPE/LLDPE silage wrap films: gas permeation properties. . Proceedings of the Agricultural Research Forum, Tullamore, 14 & 15 March, 2005, p78.
Laffin, C., McNally, G.M., Forristal, P.D., O'Kiely, P. and Small, C.M. (2005). The effect of uni-axial stretching on the gas permeation properties of LDPE/LLDPE silage wrap film. Proceedings of the ANTEC Conference, Boston, Massachusetts USA, May 2005. p143-147.
McEniry, J., O'Kiely, P., Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2006). Changes in bacterial community structure in a grass silage fermentation. Proceedings of 11th International Symposium on Microbial Ecology, 20 – 25 August, Vienna, Austria, Abstract # 1488, p304.
McEniry, J., O'Kiely, P., Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2006). Bacterial community changes in silage fermentation. Proceedings of 158th meeting of the Society for General Microbiology, University of Warwick, 3-6 April, p52.
McEniry, J., O'Kiely, P., Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2006). Bacterial community changes in a silage fermentation. Proceedings of the Society for General Microbiology, Irish Branch Symposium, Microbial Bioconversions: Biocatalysis and Biodegradation, UCD Dublin, 31 August and 1 September, p 22.
McEniry, J., O'Kiely, P., Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2006). The microbiological and chemical composition of silage over the course of fermentation in round bales relative to that of comparable precision-chop silage. Proceedings of the Agricultural Research Forum, Tullamore, 15 & 16 March, 2006, p74.
McEniry, J., O'Kiely, P., Clipson, N.J.W., Forristal, P.D. and Doyle, E.M. (2006). Bacterial community changes in silage over the course of fermentation in round bales relative to that of comparable precision-chop silage. Proceedings of the Agricultural Research Forum, Tullamore, 15 & 16 March, 2006, p118.
McEniry, J., O'Kiely, P., Clipson, N.W.J., Forristal, P.D. and Doyle, E.M. (2005). Microbiological composition of silage: the effect of wilting, chopping, compaction and anaerobiosis. . Proceedings of the Agricultural Research Forum, Tullamore, 14 & 15 March, 2005, p75.
McEniry, J., O'Kiely, P., Clipson, N.W.J., Forristal, P.D. and Doyle, E.M. (2005). Bacteria and yeast in round bale silage on a sample of farms in County Meath, Ireland. Proceedings of the 14th International Silage Conference (and a satellite workshop of the 20th International Grassland Congress) (eds. R.S. Park and M.D. Stronge), Belfast, 3 to 6 July 2005. Wageningen Academic Publishers, p248.
McEniry, J., O'Kiely, P., Clipson, N.W.J., Forristal, P.D. and Doyle, E.M. 2007 Investigating the factors involved in baled silage fermentation using conventional and molecular techniques. School of Biology and Environmental Science, Postgraduate Seminar day, 10 May, Agriculture and Food Science Centre, University College Dublin, absract # 2, page 4.
O'Brien, M., Nielsen, K.F., O'Kiely, P., Forristal, P.D., Fuller, H. & Frisvad, J.C. (2007). Mycotoxins and other secondary metabolites produced by *Penicillium* spp. isolated from baled grass silage in Ireland. Proceedings of the Agricultural Research Forum, 12 & 13 March, 2007, Tullamore, p102.
O'Brien, M., Nielsen, K.F., O'Kiely, P., Forristal, P.D., Fuller, H. & Frisvad, J.C. (2007). Toxin-producing fungi in baled grass silage in Ireland. Proceedings of Irish Fungal Meeting, Tues. 19 June, 2007, National University of Ireland, Maynooth. (Poster 28) 1 page.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2005). Fungal contamination of baled grass silage at feedout on farms during November 2003 to March 2004. Proceedings of the Irish Plant Scientists Association meeting, UCD Belfield, 30 March – 1 April, p9 (abstract).

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2005). *Schizophyllum* on baled grass silage in Ireland: national farm survey 2004. Proceedings of the 14th International Silage Conference (and a satellite workshop of the 20th International Grassland Congress) (eds. R.S. Park and M.D. Stronge), Belfast, 3 to 6 July 2005. Wageningen Academic Publishers, p249.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2005). National survey to establish the extent of visible mould on baled grass silage in Ireland and the identity of the predominant fungal species. Proceedings of the 14th International Silage Conference (and a satellite workshop of the 20th International Grassland Congress) (eds. R.S. Park and M.D. Stronge), Belfast, 3 to 6 July 2005. Wageningen Academic Publishers, p252.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2005). The extent and identity of fungi on baled silage at feedout on farms in the midlands. . Proceedings of the Agricultural Research Forum, Tullamore, 14 & 15 March, 2005, p74.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2006). Viable fungal spores in big-bale silage. Proceedings of the 16th Irish Environmental Researchers Colloquium (Ed. T.Wilkinson), 27-29 January, UCD, p65.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2006). Quantification and identification of fungal propagules in bales of grass silage produced using standard farm procedures. Proceedings of the Agricultural Research Forum, Tullamore, 15 & 16 March, 2006, p117.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2007). Fungal contamination baled grass silage in Ireland. Proceedings of Irish Fungal Meeting, Tues. 19 June, 2007, National University of Ireland, Maynooth. (Talk 8) 1 page.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller,H. (2005). Fungal propagules in clean bales of silage. Proceedings of the Irish Plant Scientists Association meeting, UCD Belfield, 30 March – 1 April, p25 (abstract).

O'Brien, M., O'Kiely, P., Forristal, P.D. and O'Mara, F.P. (2005). Numbers of mould and yeast propagules in baled silage devoid of visible fungal growth. . Proceedings of the Agricultural Research Forum, Tullamore, 14 & 15 March, 2005, p112.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2004). Pilot survey to establish the occurrence and identity of visible fungi on baled silage. Proceedings of the Agricultural Research Forum, Tullamore, 1-2 March, p50.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2004). The development of an optimal system for sampling the outer horizon of silage bales for fungal propagules. Proceedings of the Agricultural Research Forum, Tullamore, 1-2 March, p94.

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2004). Mould growth on baled grass silage. Irish Plant Scientists Association meeting, Queens University Belfast. 21-23 April, 1page [abstract].

O'Brien, M., O'Kiely, P., Forristal, P.D. and Fuller, H. (2004). Occurrence of Penicillium roqueforti and other fungi on baled grass silages in Ireland. Proceedings of the British Mycological Society annual scientific meeting (Fungi and the environment), University of Nottingham, Sept. 2004, p37.