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The role of indigenous yeasts in traditional Irish cider fermentations

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ABSTRACT

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Aims: To study the role of the indigenous yeast flora in traditional Irish cider fermentations.

Methods and Results: Wallerstein laboratory nutrient agar supplemented with biotin, ferric ammonium citrate, calcium carbonate and ethanol was employed together with PCR-restriction fragment length polymorphism analysis of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5·8S rRNA gene in the identification of indigenous yeasts at the species level, from traditional Irish cider fermentations. By combining the molecular approach and the presumptive media it was possible to distinguish between a large number of yeast species, and to track them within cider fermentations. The Irish cider fermentation process can be divided into three sequential phases based on the predominant yeast type present. *Kloeckera/Hanseniaspora uvarum* type yeasts predominate in the initial 'fruit yeast phase'. Thereafter *Saccharomyces cerevisiae* type yeast dominate in the 'fermentation phase', where the alcoholic fermentation takes place. Finally the 'maturation phase' which follows, is dominated by *Dekkera* and *Brettanomyces* type yeasts. *H. uvarum* type yeast were found to have originated from the fruit. *Brettanomyces* type yeast could be traced back to the press house, and also to the fruit. The press house was identified as having high levels of *S. cerevisiae* type yeast. A strong link was noted between the temperature profile of the cider fermentations, which ranged from 22 to 35°C and the yeast strain population dynamics.

Conclusions: Many different indigenous yeast species were identified. The mycology of Irish cider fermentations appears to be very similar to that which has previously been reported in the wine industry.

Significance and Impact of the Study: This study has allowed us to gain a better understanding of the role of indigenous yeast species in 'Natural' Irish cider fermentations.

Keywords: *Brettanomyces/Dekkera*, cider, fermentation dynamics, indigenous yeast, *Saccharomyces cerevisiae*.

INTRODUCTION

Cider is a common alcoholic beverage in a number of different European countries. In many regions of countries such as France, Spain, Ireland and Slovenia cider is still produced by a natural fermentation process, involving naturally occurring indigenous yeast species (Michel *et al.* 1988; Vidrih and Hribar 1999). These traditional type fermentations typically use wooden mills to crush the fruit and batch mechanical presses to extract the juice. Fermentations are then performed in wooden casks with no temperature control. Wild microflora, which typically originate from the fruit or from the surfaces of the processing equipment typically, perform the alcoholic fermentations. However the use of these methods often causes uncontrolled fermentations and subsequent variation in the final quality of the cider.

The mycological-based conversion of both grape must into wine, and apple must into cider is known to involve a

**This manuscript is dedicated to the memory of Bob Davenport.*

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complex mixture of many different species. It is clear from previous studies that there is great similarity between the yeast involved and the population dynamics within both the wine and cider fermentation processes (Beech 1972, 1993; Davenport 1974; Williams 1974; Salih and Drilleau 1988; Fleet 1990; Vaughan-Martini and Martini 1995; Le Quere and Drilleau 1996; Laplace *et al.* 1998; Mortimer and Polsinelli 1999). The taxonomic investigation of the yeasts involved in both processes was initially carried out using conventional physiological and biochemical methods as described by Krejer-van Rij (1984) and Barnett (1992). While these methods provide valuable information they are labour-intensive and the results can often be misleading due to their dependency on gene expression, which can be environmentally influenced (Možina and Raspor 1997). Because of this over the last 15 years molecular methods have been adopted in the mycological taxonomy of wine yeast resulting in greater reproducibility and discriminatory power, together with more rapidly obtainable results (White *et al.* 1990; Vezinhet *et al.* 1992; Guillamon *et al.* 1998; Deak 1999; Dlauchy *et al.* 1999; Sabate *et al.* 2002). In contrast, little molecular work has been carried out on the yeast involved in cider fermentations.

The present study centred on a cider fermentation facility in Ireland, involving a process, which has changed little since the plant was originally set up in 1935. The press house consists, internally of wooden structures with a porous plaster-like material on the walls. The cider press is a traditional rack and frame press. Apple pomace is wrapped in cheesecloths, a wooden rack is placed over it and the process is repeated for 12 consecutive layers. This is referred to as a cheese, which is constructed on wooden trolleys, called bogeys, which are pushed from press to press. The cheese is then pressed by hydraulic rams and the fresh apple juice (also referred to as must) flows into underground ceramic pipes that lead to a holding tank.

During the pressing season, which typically lasts from early September to late December, the fresh juice is lightly sulphited (<8 ppm free SO_2) and pumped into oak vats that vary in size from 45 000 to 90 000 l. The fermentation typically starts within 24 h and as soon as the cider has reached attenuation (i.e. a specific gravity of <0·999) it is racked to stainless steel maturation vats for up to 18 months. In this study, we conducted a comprehensive mycological profile of the entire microbial ecosystem from the press house and its utensils to the vat house, together with the sampling of selected natural fermentations. These fermentations are termed 'natural' in that they do not involve the addition of yeast starter cultures. This allowed us to determine the origins and populations of the different yeast species within this ecosystem therefore allowing us to obtain a more complete understanding of this apple juice fermentation process.

Initially yeast isolates were presumptively identified using morphological identification on Wallerstein laboratory nutrient (WLN) agar supplemented with selective reagents due to the large number of yeast isolates which we encountered. The results obtained were subsequently confirmed using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5·8S rRNA gene (Esteve-Zarzoso *et al.* 1999), and by comparison with patterns obtained using standard NCYC yeast strain types. Using these methods it was possible for us to determine the origin of the predominant yeast species present in a typical cider fermentation and to follow their growth profiles throughout both the fermentation and maturation processes.

MATERIALS AND METHODS

Media and culture conditions and sampling procedures

Sterile cotton swabs were used to swab the press house's walls, floors, timber support beams, wooden conveyors, cheesecloths, wooden racks and wooden bogeys. The oak vats in the press house were also swabbed in a similar manner. Swab samples were taken in quadruplicate. One set of swab samples were sonicated in 1% saline solution and serial dilutions were spread plated on the differential media WLN agar supplemented with 0·002% D-biotin and 0·05% NH_4Fe ($C_6H_5O_7$) (ferric ammonium citrate) and 0·5% $CaCO_3$, WLN supplemented with 10 and 100 ppm actidione, and WLN-E containing 10% v/v ethanol to give a plate count of between 30 and 300 colonies. The remaining swab samples were incubated over night in three different selective basal media broths containing sterilized apple juice supplemented with 0·5% yeast extract and 0·5% peptone to which either 10% ethanol and 10 ppm actidione had been added respectively. No additions were made to the final sets of broths, which served as a control. After incubation at 25°C for 48 h these broths were subsequently plated out on WLN, WLD (10 ppm cyclohexamide), WLD (100 ppm cyclohexamide) and WLN-E as described above. Sections (4 cm^2) were also taken from 20 different cheesecloths, together with 20 pieces of wood, each approx. 4 × 1 cm in size; and sonicated in 10 ml of 1% saline solution. These samples were plated out on the differential WLN, WLD (10 ppm cyclohexamide), WLD (100 ppm cyclohexamide) and WLN-E media as described above.

Mycological profile of apples

Twelve apples were randomly chosen and aseptically taken from the apple storage silos within the cider factory, at different times throughout the apple harvest season. These

were then macerated and the must extracted within a sterilized cheesecloth pouch contained within a sterile plastic bag. Serial dilutions of the fresh must were spread plated on the differential media WLN, WLD (10 ppm cyclohexamide), WLD (100 ppm cyclohexamide) and WLN-E, to give a plate count of between 30 and 300 colonies.

Cider fermentations

In this study, all fermentation were carried out in similar-sized fermentation vessels, with no temperature control and there was little variation between the fresh musts used, due to the wide variety of apples processed on a daily basis (data not shown). Samples were periodically taken from these fermentations, during both the fermentation and maturation stages. Serial dilutions of samples were spread plated onto the differential media WLN, WLD (10 ppm cyclohexamide), WLD (100 ppm cyclohexamide) and WLN-E, to give plate counts of between 30 and 300 colonies (within 1 h of sampling) and incubated at 25°C for 5 days. Fifty random isolates were taken from each plate corresponding to each sample point (Snedecor and Cochran 1956). These isolates were then purified, streaked on YPD (4% glucose, 0·5% yeast extract, 0·5% peptone and 2% technical agar) grown at 25°C for 72 h and stored at 4°C for subsequent analysis to determine the mycological profiles of the yeast in each fermentation.

PCR-RFLP analysis of yeast DNA

The PCR was performed on DNA from yeast isolates as previously described by Esteve-Zarzoso *et al.* (1999). Direct colony PCR reactions were carried out in 100-μl volumes. The PCR reaction mix contained 0·5 μM of the ITS1 primer (5′-TCCGTAGGTAACCTGCGG-3′) and 0·5 μM of the ITS 4 primer (5′-TCCTCCGCTTATTGATATGC-3′), 0·2 mM each of dATP, dCTP, dGTP and dTTP, 1·5 mM $MgCl_2$, 1X NH_4 buffer (Bio-Line, London, UK) and 1 U *Taq* polymerase (Bio-Line). PCR amplification was performed in the Peltier PTC-200 programmable thermal controller (M.J. Research, Watertown, MA, USA). The PCR conditions were 95°C for 15 min, 40 cycles of 94°C for 1 min (denaturing), 55°C for 2 min (annealing), 72°C for 2 min (elongation) followed by a final elongation of 72°C for 10 min. A quantity of 10 μl of each reaction was separated out on 1·4% (w/v) agarose gels in TAE buffer, and 20 μl of the PCR products were then digested with *Cfo*I, *Hae*III and *Hin*fI endonucleases and separated on 3% agarose gels. Gels were stained with ethidium bromide and visualized under u.v. light. Electrophoretic patterns were compared with those obtained from standard yeasts strains obtained from the NCYC: *Metschinikowia pulcherrima* NYC 373, *Pichia anomola* NCYC 750, *Brettanomyces anomalus* NCYC 749, *B. bruxellensis* NCYC 2818, *Debaromyces polymorphus* NCYC 947, *H. uvarum* NCYC 2739, *P. fermentas* NCYC 562, *P. guilliermondii* NCYC 443, *Saccharomycodes ludwigii* NCYC 734 and *Saccharomyces cerevisiae* NCYC 76.

Physico-chemical analysis

Samples for chemical analysis were taken in triplicate and stored at −18°C. The temperature of each fermentation was taken at each sampling point. Ethanol, temperature and sugar content determinations were carried out using standard AICV (L'Association des Industries des Cidres et Vins de fruits de l'U.E.) methodology. Sugar utilization was determined by monitoring the decrease in sugar levels between each sample point using the Anton Paar DMA 5000 Density Meter® (Anton Paar Ltd, Herts, UK). Ethanol % v/v determination of the distillate from cider samples was also performed using the Anton Paar DMA 5000 Density Meter®.

RESULTS

Given that our overall aim was to isolate, identify and enumerate the predominant indigenous yeast species present within the existing cider fermentation facility; from the press house to the vat house together with those that were present in various natural fermentations, it was clear that samples would initially need to be plated on various yeast selective media.

In this regard, WLN agar supplemented with differential reagents proved extremely useful in the isolation and initial presumptive identification of different yeast species, from this large number of samples (Table 1). It was particularly useful in differentiating between *Metschnikowia* and *Hanseniaspora/Kloeckera* type species with *Metschnikowia* species producing a distinct red pigment in media supplemented with D-biotin and ferric ammonium citrate, while *Hanseniaspora/Kloeckera* types displayed a characteristic green colour. *Brettanomyces/Dekkera* type species were also easily distinguishable displaying a yellow halo which was clearly visible against the blue media background. The WLN media supplemented with 10% ethanol (WLN-E) also proved useful in the isolation of *S. cerevisiae* species. We confirmed the identity of these yeasts using the ITS1 and ITS4 primer pair, which amplified the region between the 18S rRNA and the 28S rRNA genes in each case. The PCR products and subsequent RFLP restriction patterns obtained for the yeast species which had been isolated on the different presumptive were then compared with those obtained using the corresponding standard NCYC yeast strains (Fig. 1a,b). The patterns obtained allowed us to identify these different yeasts strains at the species level and permitted us to subsequently use this method routinely to follow these different yeast species throughout different fermentations (Table 2).

Table 1 Presumptive identification of cider yeast

Yeast genus	Growth 10 ppm cyclohexamide	Growth 100 ppm cyclohexamide	Colour on ferric ammonium citrate and D-biotin	Colour on WLN	Optimum growth time at 25°C (days)	Shape of cells	Type of budding
Hanseniaspora/ Kloeckera	+	–	Green	Green	3–5	Apiculate (lemon-shaped cell)	Bipolar
Metschnikowia	+/–	–	Metallic red	Dark green	3–5	Small round cells often found in bundles of three	Multilateral
Debaryomyces	–	–	Predominantly white	Predominantly white	3–5	Mother cell often surrounded by two daughter cells	Multilateral
Dekkera/ Brettanomyces	+	+	Predominantly green surrounded by a yellow halo	Predominantly green with light blue head	3–14 (reduced in the presence of 100 ppm thiamine)	Ogival ended rectangular shaped with budding sometimes taking place at a corner of the rectangular end	Multilateral
Saccharomycodes ludwigii	+/–	–	Shiny white colonies	Shiny white colonies	3–5	Large apiculate (bowling pin-shaped cell)	Bipolar
Saccharomyces cerevisiae	–	–	Variable	Variable	3–5	Round to oval-shaped cells with a characteristic granular appearance in the middle	Multilateral

Table 3 shows the different sources of yeast in the traditional cider-making process. Quantitative analysis of *Saccharomyces* type species was carried out in the press house and its utensils. Most areas within the press were colonized with *Saccharomyces* type species ranging from $5{\cdot}0 \times 10^2$–$4{\cdot}2 \times 10^4$ CFU cm^{-2}. The fresh must was also a source of yeast with total yeast count varying depending on the quality of fruit. *Hanseniaspora/Kloeckera* type species were the major yeast species found in fresh must, typically accounting for over 80% of the total. The ratio of *Hanseniaspora/Kloeckera* type species to *S. cerevisiae* type species was also strongly influenced by the quality of the apples. In very bruised and soiled apples the population of *S. cerevisiae* type species in the must was often as high as 5×10^6 CFU ml^{-1}, in contrast to the typical population of 3×10^3 CFU ml^{-1} found on better quality apples. The other major source of yeast was the wash water, with yeast levels here depending on how often the water was recycled. Other yeast that were found to be present, but which were not quantitatively isolated included *Brettanomyces/Dekkera*, *Saccharomycodes ludwigii*, *Pichia*, *Debaryomyces* and *Metschnikowia* type species. In the vat house, the predominant yeast isolated outside of the fermentation season was *Brettanomyces/Dekkera* type species.

Yeast profiles within natural fermentations

A total of 12 separate fermentations were profiled during the course of this study, data for two of which are presented here, given that they are representative of the different times of the fermentation season. An 'Early Fermentation' (Fig. 2a,b) took place in mid-September when the ambient temperatures are typically around 20°C, with the temperature of the fresh juice being usually between 14 and 16°C. Fresh must at this time of year has the heaviest yeast load of the season. This combined with initial high must temperatures and the lack of cooling capacity from ambient temperatures resulted in a very rapid (12–15 days) fermentation with temperatures reaching up to 34–36°C. The initial yeast count was $6{\cdot}0 \times 10^6$ CFU ml^{-1} of which over 90% was composed of *Hanseniaspora/Kloeckera* type yeast. In the first 24 h there was a short lag phase followed by a rapid utilization of sugars coinciding with an increase in fermentation temperatures from 16 to 20·4°C (Fig. 2a). There was an accompanying exponential growth in the number of *Hanseniaspora/Kloeckera apiculata* and *S. cerevisiae* type yeast species. As the alcohol level rises to above 4% alcohol, on day 3 the levels of *Hanseniaspora/K. apiculata* type species decreased. *Hanseniaspora* and other strains were no longer detected after day 12 with the WLD plate

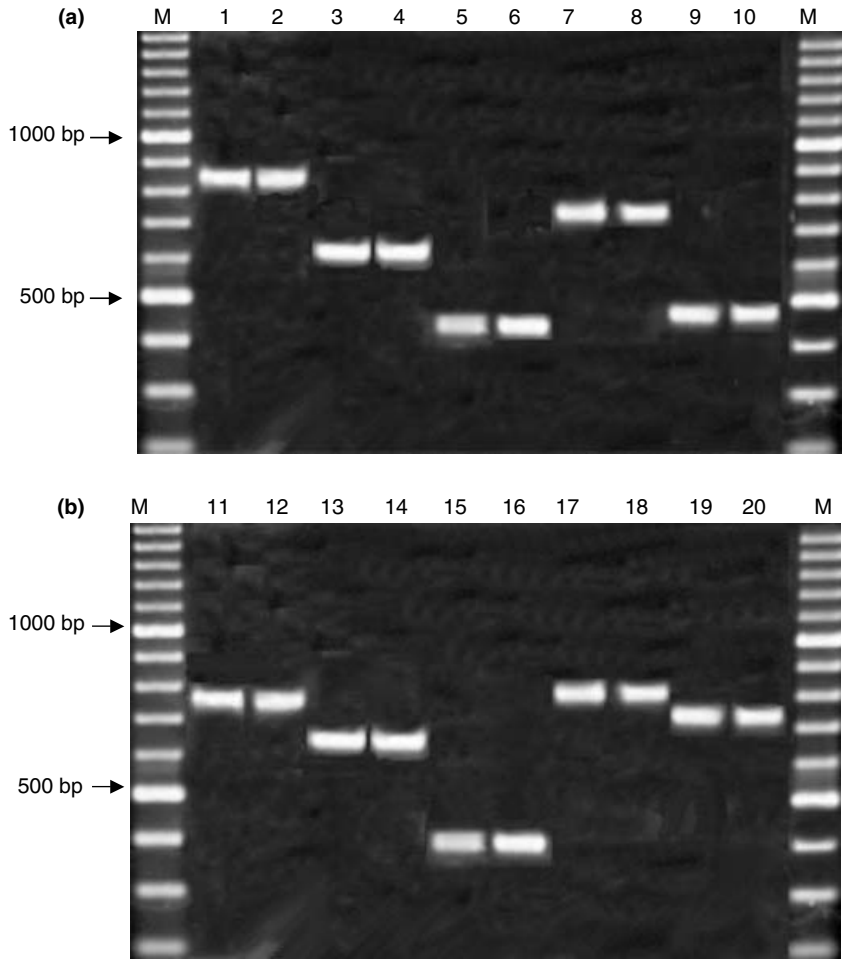

Fig. 1 (a) PCR products obtained using the primers ITS1 and ITS4. Lanes 1–10: (1) *Saccharomyces cerevisiae* NCYC 76, (2) *Saccharomyces cerevisiae* BM1, (3) *Pichia guilliermondii* NCYC 443, (4) *Pichia guilliermondii* BM5, (5) *Pichia fermentas* NCYC 562, (6) *Pichia fermentas* BM43, (7) *Hanseniaspora uvarum* NCYC 2739, (8) *Hanseniaspora uvarum* BM34, (9) *Brettanomyces bruxellensis* NCYC 2818, (10) *Brettanomyces bruxellensis* BM 23. M is a 100-bp ladder. (b) PCR products obtained using the primers ITS1 and ITS4. Lanes 11–20: (11) *Saccharomycodes ludwigii* NCYC 734, (12) *Saccharomycodes ludwigii* BM 26, (3) *Pichia anomola* NCYC 750, (14) *Pichia anomola* BM19, (15) *Metschinikowia pulcherrima* NYC 373 (16) *Metschinikowia pulcherrima* BM 33, (17) *Brettanomyces anomalus* NCYC 749, (18) *Brettanomyces anomalus* BM11, (19) *Debaromyces polymorphus* NCYC 947, (20) *Debaromyces polymorphus* BM53, M is a 100-bp ladder

used to detect *Hanseniaspora* being colonized predominantly by *Brettanomyces/Dekkera* type yeast.

At this stage *Saccharomyces* type yeast began to dominate, with the fermentation peaking typically at $8{\cdot}3 \times 10^8$ CFU ml^{-1} on day 5 (Fig. 2a). This was followed by a marked decrease in the population, which dropped to $1{\cdot}3 \times 10^7$ CFU ml^{-1} at attenuation, and to $5{\cdot}0 \times 10^6$ CFU ml^{-1} by day 18. As the numbers of *S. cerevisiae* type species diminish *Brettanomyces/Dekkera* type yeast species began to be detected again on day 12, with their overall numbers increasing from 11% of the total yeast population to over 90% of the population by day 22 (Fig. 2a). By day 25 the fermented cider was racked and allowed to mature for up to 18 month. During this period *Brettanomyces* were the sole yeast species detected. A random sampling of other maturation vats from each of the other 10 fermentations studied, also found *Brettanomyces/Dekkera* to be the dominant yeast species present at this stage, with levels $>3 \times 10^3$ CFU ml^{-1} being detected at up to 9 months of maturation.

There appears to be a marked difference in the yeast profiles which we observe in 'later fermentations' (Fig. 3-a,b) which are conducted in December; than those which were undertaken earlier in the season. The ambient temperature in December is typically 10–12°C with fresh must temperatures rarely rising above 12°C (Fig. 3b). The overall levels of yeast present is also quite different with higher yeast levels in the must earlier in the early fermentations ($5{\cdot}95 \times 10^6$ CFU ml^{-1}) (Fig. 2a) with levels of $2{\cdot}5 \times 10^6$ CFU ml^{-1} in the later fermentation (Fig. 3a). This in combination with the lower overall ambient temperatures resulted in a slower fermentation which takes over 40 days to reach attenuation. In the early season fermentation, we noticed a sharp rise in *S. cerevisiae* type species followed by an equally sharp decrease (Fig. 2a). In the later fermentations with temperatures generally not exceeding 24°C the total yeast counts were not as high, reaching levels of $2{\cdot}1 \times 10^7$ CFU ml^{-1} as opposed to $8{\cdot}34 \times 10^8$ CFU ml^{-1} in the typical September fermentation. There was also a far shorter exponential growth phase

Table 2 Identification of the predominant yeast species found in this study (designated BM); according to size of the PCR product amplified from the 5·8S-ITS regions and subsequent fragments following digestion with the restriction endonucleases *Cfo*I, *Hae*III and *Hin*fI

Species	Amplified product (ITS1 & IT4 primers)	Restriction fragments		
		*Cfo*I*	*Hae*III*	*Hin*fI*
Saccharomyces cerevisiae NCYC 76	880	385 + 365	325 + 230 + 170 + 125	360 + 350 + 160
Saccharomyces cerevisiae BM1	880	385 + 365	325 + 230 + 170 + 125	360 + 350 + 160
Hanseniaspora uvarum BM34	750	320 + 310 + 105	750	350 + 200 + 180
Hanseniaspora uvarum NCYC 2739	750	320 + 310 + 105	750	350 + 200 + 180
Brettanomyces bruxellensis BM 23	485	250 + 140 + 90	375 + 95	270 + 215
Brettanomyces bruxellensis NCYC 2818	485	250 + 140 + 90	375 + 95	270 + 215
Brettanomyces anomalus BM11	800	340 + 340 + 120	800	360 + 190 + 160 + 80
Brettanomyces anomalus NCYC 749	800	340 + 340 + 120	800	360 + 190 + 160 + 80
Metschinikowia pulcherrima BM 33	400	205 + 100 + 95	280 + 100	200 + 190
Metschinikowia pulcherrima NCYC 373	400	205 + 100 + 95	280 + 100	200 + 190
Debaromyces polymorphus BM53	730	300 + 200 + 180 + 100	650 + 80	310 + 200 + 140 + 100
Debaromyces polymorphus NCYC 947	730	300 + 200 + 180 + 100	650 + 80	310 + 200 + 140 + 100
Pichia fermentas BM43	450	170 + 100 + 100 + 80	340 + 80	250 + 200
Pichia fermentas NCYC 562	450	170 + 100 + 100 + 80	340 + 80	250 + 200
Pichia guilliermondii BM5	625	300 + 265 + 60	400 + 115 + 90	320 + 300
Pichia guilliermondii NCYC 443	625	300 + 265 + 60	400 + 115 + 90	320 + 300
Pichia anomola BM 19	650	575	600 + 50	310 + 310
Pichia anomola NCYC 750	650	575	600 + 50	310 + 310
Saccharomycodes ludwigii BM 26	750	360 + 350	700	450 + 275
Saccharomycodes ludwigii NCYC 734	750	360 + 350	700	450 + 275

*Values refer to the number of base pairs per fragment.

Table 3 Yeast population found in fresh must, wash water and the press house and its utensils. NA denotes not analysed

Sample point	*Saccharomyces cerevisiae* (CFU cm^{-2})	Total yeast (CFU ml^{-1})
Fresh must channels	$3{\cdot}2 \times 10^4$	NA
Pomade conveyor	$3{\cdot}7 \times 10^3$	NA
Bogie	$3{\cdot}5 \times 10^4$	NA
Wall	$3{\cdot}7 \times 10^4$	NA
Cheesecloths	$5{\cdot}0 \times 10^2$	NA
Slats	$4{\cdot}2 \times 10^4$	NA
Fresh must (good quality apples)	NA	$2{\cdot}0 \times 10^4$
Fresh must (poor quality apples)	NA	$5{\cdot}0 \times 10^6$
Wash water	NA	$5{\cdot}0 \times 10^3$–$2{\cdot}0 \times 10^5$

involving *S. cerevisiae* type species, corresponding with a reduced utilization of sugars (Fig. 3b), while the overall levels of *Saccharomyces* type yeasts remained relatively constant throughout the remainder of the fermentation (days 15–35) (Fig. 3a). Finally *Brettanomyces* species were not detected until day 100 (data not shown), which is a much later stage in the fermentation process than is the case in the early season fermentations.

DISCUSSION

The main aim of this study was to isolate identify and enumerate the predominant indigenous yeast species present within an existing cider fermentation facility. The predominant yeast species identified were *Metschinikowia pulcherrima*, *P. anomola*, *B. anomalus*, *B. bruxellensis*, *Debaromyces polymorphus*, *H. uvarum*, *P. fermentas*, *P. guilliermondii*, *Saccharomycodes ludwigii* and *S. cerevisiae*.

With respect to the source of the yeast species, which we identified, there appears to be two main sources for the fermentative *S. cerevisiae* type yeasts. One source was the apples themselves, with high numbers being observed (2×10^4–5×10^6 CFU ml^{-1}) this is in contrast to previous reports where little or no yeast were found on skins of good quality apples or grapes (Davenport 1974; Vaughan-Martini and Martini 1995), but other groups have reported high levels of *S. cerevisiae* type yeast on damaged or mummified fruit (Mortimer and Polsinelli 1999; Sniegowski *et al.* 2002). Indeed in recent studies on the ecology of wine, it is clear that many of the yeasts involved originate from either the grapes or are associated with the surfaces of the winery equipment and environment (Fleet 2001; Fleet 2003). Thus it is perhaps not surprising that in our case the other main source of *S. cerevisiae* type yeasts appears to be the process utensils, which were found to have substantial yeast

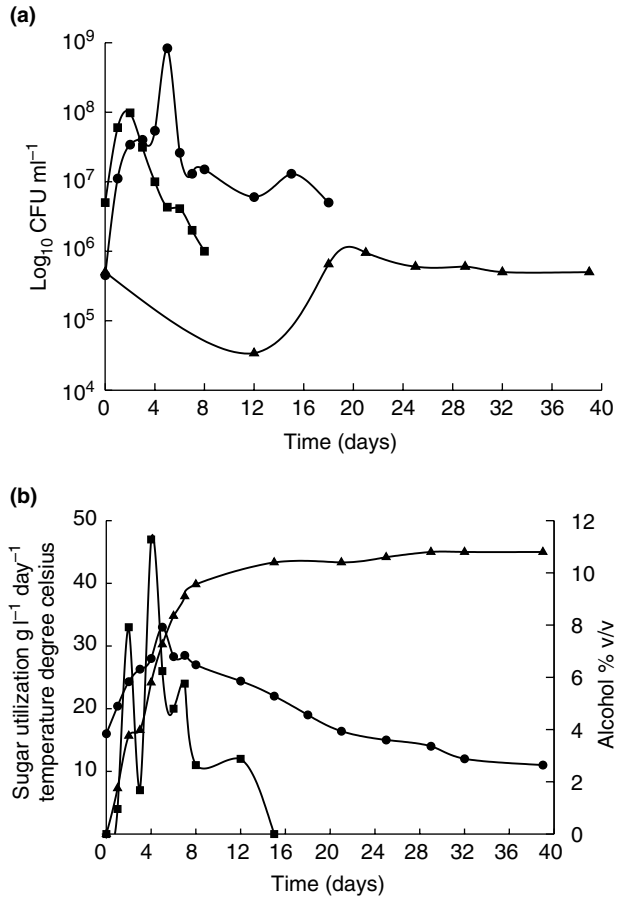

Fig. 2 (a) Typical mycological profile of an early fermentation: *Hanseniaspora/Kloeckera* (■), *Saccharomyces cerevisiae* (●), *Brettanomyces/Dekkera* (▲). (b) Typical physiochemical profile of an early fermentation: temperature (°C), ■; sugar utilization (g l^{-1} day^{-1}), ●; alcohol (%, v/v), ▲

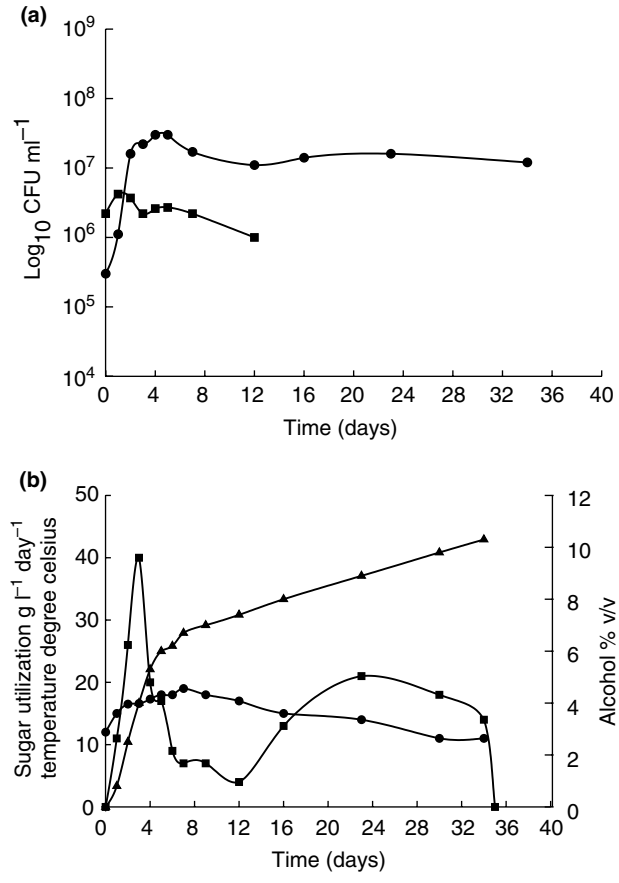

Fig. 3 (a) Typical mycological profile of a later fermentation: *Hanseniaspora/Kloeckera* (■), *Saccharomyces cerevisiae* (●). (b) Typical physiochemical profile of a later fermentation: temperature (°C), ■; sugar utilization (g l^{-1} day^{-1}), ●; alcohol (%, v/v), ▲

populations even in the month of June, which is approx. 6 months since the last pressing. Yeast levels of $3{\cdot}2 \times 10^4$ CFU cm^{-2} were also observed on the ceramic surface of the fresh must channels, even in the off-season and given that these surfaces would not have been exposed to any fresh must since December of the previous fermentation season, they seem to retain a large yeast population on their surfaces. This is in contrast to the cheesecloths which might have been expected to have a high yeast content due to the constant direct contact with apple pomace and juice; however, relatively low yeast counts were observed. This may to be due to the fact that they are boiled in December in 2% sodium carbonate and are left to dry in the rafters of the press house. *H. uvarum* type yeast were found to have originated from the fruit while the *Brettanomyces* type yeast which appear to dominate the 'Maturation phase' could be traced back to press house, and also to the fruit. The yeast populations found in both the press house and the vat house during the off-season could be considered to be the 'resident yeast' within the plant, having the ability to survive from one season to another without any contact with fresh must. The traditional press house and vat house date back to the 1880s and the surfaces of their interior walls are composed of old damp plaster that is very difficult to sanitize, and would thus provide an ideal environment for the survival of a wide range of yeast species from one year to the next. In similar studies conducted in wineries *S. cerevisiae* involved in the wine fermentations have been shown to be present on cellar walls (Martini 1993). This is in marked contrast to what we observed in the new modern press house with purpose designed nonporous surfaces. This press house is regularly sanitized throughout the pressing season and is thoroughly cleaned at the end of the pressing season. This was mycologically profiled in an identical manner to the old press house; with very few yeast being found throughout both the fermentation and off-seasons (data not shown). Interestingly fermentations carried out using juice from this new press house, in stainless steel vats; periodically results in

incomplete fermentations with inconsistent flavour profiles, indicating the importance of the indigenous microflora present in the traditional press house.

We found a sequential substitution of a variety of different yeast species throughout the cider-making process. A similar phenomenon has also been reported by several authors in similar studies which have been undertaken in the wine industry (Phaff and Amerine 1979; Heard and Fleet 1985; Fleet 1990, 1999; Boulton *et al.* 1995; Vaughan-Martini and Martini 1995; Mortimer and Polsinelli 1999). This allowed us to divide the process into three distinct mycological phases based on the predominant yeast species present. In the initial 'fruit yeast phase' *Hanseniaspora/K. uvarum* type yeasts were the predominant species; however, we found that a large population of *S. cerevisiae* type yeast was also present. The 'fruit yeast phase' yeast were quickly substituted with the strong fermenting *S. cerevisiae* type yeast which dominate the 'fermentation phase', where the alcoholic fermentation takes place. This replacement of non-*Saccharomyces* yeasts which grow well during the early stages of fermentation but which are subsequently replaced by *Saccharomyces* yeasts, which are more ethanol tolerant is also a common phenomenon in natural wine fermentations (Fleet and Heard 1993). This replacement of *Hanseniaspora/K. uvarum* type yeast with *Saccharomyces* type yeast has as yet however not been fully explained. Factors including depletion in oxygen levels, production of CO_2, differences in specific growth rates, differing sugar uptake capabilities, essential nutrient availability, inter-specific competition, cell death, flocculation and/or natural sedimentation characteristics are likely to be involved (Fleet 2003). Finally the 'maturation phase' which follows, is dominated by *Dekkera* and *Brettanomyces* type yeasts. The presence of this yeast has also been reported in French cider (Le Quere and Drilleau 1996) and in lambic beer fermentations and is often favourable in certain young red wines (Boulton *et al.* 1995).

A strong link was noted between the temperature profile of the cider fermentations, and the yeast population dynamics of the predominant yeast species, present within the fermentations.

Fermentation temperature is well known to markedly influence both the overall fermentation performance and the organoleptic properties of wines (Fleet and Heard 1993). Early season fermentations when ambient temperatures were higher with fresh must temperatures of 14–16°C, which in addition to the higher yeast numbers present, seemed to result in very rapid fermentation (Fig. 2a,b). The rapid increase in sugar utilization, temperature rise and ethanol production observed between days 4 and 8 (Fig. 2b) was accompanied by rapid growth of *Saccharomyces* species during the same period (Fig. 2a). Later in the production season at lower temperatures (which can sometimes fall below 0°C), initial yeast growth was suppressed and fermentation temperatures never rose above 24°C; resulting in much longer fermentations with very different mycological profiles (Fig. 3a). In these fermentations which had lower initial overall numbers of yeasts, there is no rapid increase in temperature; due in part to both the lower yeast load and to the lower ambient temperatures. In contrast to the earlier fermentations, following an initial growth phase involving *Saccharomyces* species between days 0 and 4 there was a relatively long stationary phase between days 5 and 35; which coincided with a initial increase in temperature from 12 to 18°C followed by a slow decrease in temperature until the end of the alcohol fermentation at day 36. Another interesting difference between the typical earlier and later season fermentations was the absence of *Dekkera/Brettanomyces* type yeasts in the later fermentations until after day 100 (data not shown), indicating a much later onset for the maturation phase.

Irrespective of the overall fermentation profile, once the alcoholic fermentation phase had been completed we found that *Dekkera/Brettanomyces* type yeasts dominated the maturation phase in every fermentation. This phenomenon has also previously been reported in French cider (Le Quere and Drilleau 1996). Given the prevalence of *Dekkera/Brettanomyces* species in samples which we isolated from the plant such as in the vat and in the press house, coupled with the fact that they were present in all of the fermentations that we monitored; we believe that these species are likely to contribute to the overall organoleptic properties of this particular cider.

Thus this work has allowed us to enumerate, isolate and identify many different indigenous yeast species which are involved in a traditional Irish cider fermentation process. The mycology of Irish cider fermentations appears to be very similar to that of the wine industry and these similarities should allow us to gain further insights into the role of these different yeast species in the production of this quite unique cider product.

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