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# Dynamics of Yeast Populations during Cider Fermentation in the Asturian Region of Spain

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**The influence of different cidermaking systems and apple mixtures on the dynamics of yeast populations in cider manufactured in Asturias (northern Spain) were studied. Results obtained in an experimental pilot plant were compared with those found in Asturian cider plants by using traditional techniques. *Saccharomyces cerevisiae* and *Kloeckera apiculata* were found in all cases.**

Natural fermentation continues to be the main method of cider fermentation carried out in Spain. The available information is insufficient to permit full understanding and control of the cidermaking process in Asturias. As a consequence, the product varies greatly and its organoleptic properties are not reproducible.

This study has been undertaken to determine some quantitative and qualitative aspects of the yeast microflora found in Asturian musts and ciders. The yeast microflora found in three Asturian cider plants ($1.5 \times 10^5$ to $6.0 \times 10^5$ liters of cider per year) and in our experimental pilot plant have been studied. In the pilot plant, we investigated two technologies and two different mixtures of apples (Fig. 1).

The effects of enzymatic and spontaneous keeving (1, 4) have been studied too (keeving is the pectin coagulation in apple must) (Fig. 1). Before fermentation, enzymatic keeving was carried out with pectin esterase (12 U of pectin methyl esterase per liter) and calcium salts (10 mM $CaCl_2 \cdot 2H_2O$) to reduce the content of solids in the must. At keeving, the pectin was converted to pectic acid, which reacted with the calcium salts to form a jelly. Enzymatic keeving was faster than spontaneous keeving (3 versus 7 days).

All experiments were performed in duplicate; two 250-liter barrels were studied in each cider plant.

In Asturian cider plants, traditional cidermaking technology employs unwashed apples and involves milling with a hammer mill, slow pressing with a batch mechanical press, and spontaneous keeving.

Yeast microflora were counted throughout the must fermentation by serial dilution in sterile peptone-water. The samples were spread on yeast malt agar with 2 ppm (wt/vol) of dactinomycin and 25 ppm (wt/vol) of aureomycin (2).

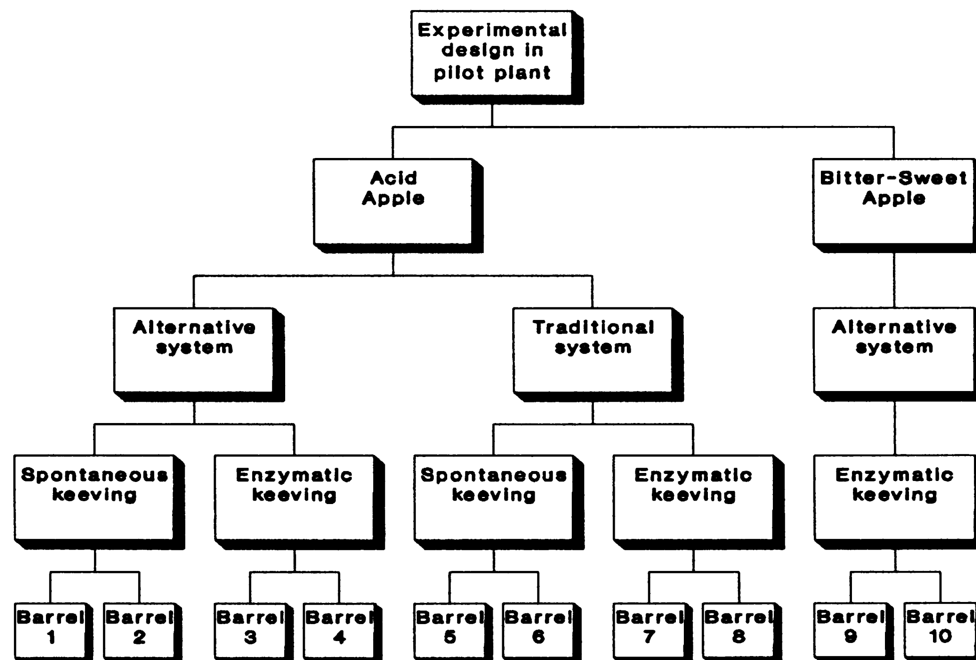


FIG. 1. Experiment in the pilot plant. We investigated two technologies, two different mixtures of apples, and the effects of enzymatic and spontaneous keeving. In the traditional system, unwashed apples, a hammer mill, and a slow press cycle (3 days) were used; in the alternative system, washed apples, a grating mill, and a fast press cycle (1 h) were used. Due to an unexpected shortage of must, barrel 2 could not be used in the experiment.

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TABLE 1. Pilot plant yeast count throughout cidermaking by using two technologies and two different mixtures of apples

| Stage | Yeast count (CFU/ml) in barrels[a]: | | | | |
|---|---|---|---|---|---|
| | 1 + 2 | 3 + 4 | 5 + 6 | 7 + 8 | 9 + 10 |
| Must | $1.31 \pm 0.02 \times 10^6$ | $1.31 \pm 0.02 \times 10^6$ | $4.45 \pm 0.17 \times 10^6$ | $4.45 \pm 0.17 \times 10^6$ | $1.12 \pm 0.01 \times 10^6$ |
| Rapid fermentation | $4.70 \pm 0.02 \times 10^7$ | $1.07 \pm 0.21 \times 10^7$ | $1.90 \pm 0.00 \times 10^7$ | $1.54 \pm 0.01 \times 10^7$ | $2.63 \pm 0.22 \times 10^7$ |
| Start slow fermentation | $4.00 \pm 0.01 \times 10^7$ | $1.28 \pm 0.06 \times 10^7$ | $1.23 \pm 0.02 \times 10^7$ | $1.23 \pm 0.02 \times 10^7$ | $1.73 \pm 0.02 \times 10^7$ |
| End slow fermentation | $3.40 \pm 0.04 \times 10^6$ | $1.99 \pm 0.05 \times 10^7$ | $3.38 \pm 0.14 \times 10^6$ | $7.24 \pm 0.04 \times 10^6$ | $9.12 \pm 0.04 \times 10^6$ |
| Malolactic fermentation | $3.30 \pm 0.03 \times 10^6$ | $3.23 \pm 0.04 \times 10^6$ | $7.07 \pm 1.20 \times 10^3$ | $8.91 \pm 0.81 \times 10^5$ | $5.01 \pm 0.04 \times 10^6$ |
| After bottling | $7.25 \pm 0.06 \times 10^3$ | $2.75 \pm 0.04 \times 10^6$ | $8.31 \pm 0.53 \times 10^2$ | $1.12 \pm 0.24 \times 10^4$ | $3.16 \pm 0.01 \times 10^4$ |

[a] As shown in Fig. 1, the apple mixtures and technologies for the barrels were as follows: 1, 2, 5, and 6, acid apples and spontaneous keeving; 3, 4, 7, and 8, acid apples and enzymatic keeving; and 9 and 10, bittersweet apples and enzymatic keeving. The alternative system was used for barrels 1 to 4, 9, and 10; the traditional system was used for barrels 5 to 8.

Yeast counts were carried out at different stages (Tables 1 and 2). Yeast strains were identified by the procedures of Kreger-Van Rij (11).

After a log transformation of microbial counts, analyses of variance were performed on data obtained at different stages of the cidermaking, with speed of pressing, keeving system, and apple mixtures as the main factors. The type of analysis performed was a repeated measurement analysis of variance (13, 18). Due to insufficient repetitions, the three-factor interactions could not be calculated. Accordingly, separate analyses were performed for the apple mixtures and for the cidermaking systems.

The analysis of time effects was performed in two different ways. First, an overall analysis was done by using the Greenhouse-Geiser adjustment (9). Second, the differences between two consecutives samples were studied for their significance by using an orthogonal contrast.

**Pilot plant.** There was a significant effect of both speed of pressing ($P = 0.03$) and keeving system ($P = 0.04$) with the mixtures of acid apples, but their interaction was not significant (Table 1). The overall evolution of yeast was found to be affected by the speed of pressing ($P = 0.04$) and by the keeving system ($P = 0.05$), but with only a marginal effect of their interaction. In addition, an analysis of differences between two consecutive samples showed that the differences between stages M1 and M2 were due to the effects of the pressing and keeving systems ($P = 0.0069$ and $P = 0.03$, respectively) and to their interaction ($P = 0.06$), but the differences between the counts in the later samples were not explained by these factors. The speed of pressing and use of the enzymatic system clearly had marked initial effects, but their influence diminished with time. Comparing yeast levels in both systems, we can conclude that the yeast population is higher when the traditional procedure is used. This can be easily explained because this system uses unwashed apples, so that the naturally occurring yeasts are not removed; slow pressing further ensures that wild strains reach the must in maximum numbers (17, 20).

With the alternative system, by using washed apples and rapid pressing, the effect of the apple mixture on yeast numbers was significant ($P = 0.01$), and even the overall evolution of the yeast population was significantly affected ($P = 0.01$) by the mixture of apples. However, the differences between the first samples (must and rapid fermentation) could not be explained by this factor (Table 1). At the end of the process of cidermaking (samples: end of slow fermentation to after bottling), there were significant differences due to the type of apple mixture. This could be explained by differences in biochemical composition, especially in levels of polyphenolic compounds. The low yeast level with bittersweet apples could also have influenced the growth of lactic acid bacteria (7, 12), which would change the properties of the cider.

**Cider plants.** Yeast evolution is a consequence of several factors: the cider plant microclimate, hygienic conditions, type of milling and pressing, and mixture of apples. All of these are uncontrolled, and as a consequence the organoleptic properties of the cider vary greatly (14), not only among cider plants but also among different barrels in the same cider plant. Only an ecological study is possible with such conditions, and it was considered inappropriate to apply a statistical analysis to these values (Table 3).

**Yeast species isolated in cidermaking.** Three hundred twenty yeast strains were identified in the pilot plant, and 240 were identified in the Asturian cider plants (560 in all). As expected, a higher specific diversity was shown in the

TABLE 2. Cider plant yeast counts throughout cidermaking by using a traditional system with slow pressing, spontaneous keeving, and acid apples

| Stage | Yeast count (CFU/ml)[a] | | | | | |
|---|---|---|---|---|---|---|
| | I.1 | I.2 | II.1 | II.2 | III.1 | III.2 |
| Must | $6.60 \times 10^6$ | $1.47 \times 10^5$ | $4.10 \times 10^7$ | $5.15 \times 10^6$ | $1.98 \times 10^5$ | $2.51 \times 10^7$ |
| End of keeving | $8.10 \times 10^6$ | $8.10 \times 10^6$ | $1.47 \times 10^7$ | $5.85 \times 10^6$ | $8.10 \times 10^6$ | $9.90 \times 10^6$ |
| Rapid fermentation | $1.03 \times 10^7$ | $8.85 \times 10^6$ | $1.28 \times 10^7$ | $1.62 \times 10^7$ | $1.42 \times 10^7$ | $1.66 \times 10^7$ |
| Start slow fermentation | $3.65 \times 10^6$ | $4.95 \times 10^5$ | $1.52 \times 10^6$ | $9.10 \times 10^5$ | $9.90 \times 10^6$ | $3.50 \times 10^4$ |
| End slow fermentation | $5.50 \times 10^4$ | $2.97 \times 10^4$ | $5.10 \times 10^5$ | $3.05 \times 10^5$ | $3.85 \times 10^5$ | $1.00 \times 10^3$ |
| Malolactic fermentation | $1.04 \times 10^5$ | $2.28 \times 10^4$ | $5.20 \times 10^5$ | $2.00 \times 10^4$ | $2.00 \times 10^4$ | $3.55 \times 10^3$ |
| After bottling | $2.91 \times 10^3$ | $9.15 \times 10^3$ | $1.51 \times 10^5$ | $2.10 \times 10^4$ | $2.25 \times 10^3$ | $9.40 \times 10^3$ |
| After bottling (3 mo) | $3.05 \times 10^4$ | $1.24 \times 10^4$ | $1.70 \times 10^4$ | $6.90 \times 10^3$ | $4.60 \times 10^2$ | $1.21 \times 10^3$ |

[a] I.1, Cider plant I, barrel 1; I.2, cider plant I, barrel 2; II.1 cider plant II, barrel 1; II.2, cider plant II, barrel 2; III.1, cider plant III, barrel 1; III.2, cider plant III, barrel 2.

TABLE 3. Yeast species identified during cidermaking in the experimental pilot plant

| Stage | Yeast percentage(s) in barrel[a]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Must | 100 (S.K.) | 100 (S.K.) | 100 (S.K.) | 10 (H.U.)<br>70 (K.A.)<br>20 (P.O.) | 10 (H.U.)<br>70 (K.A.)<br>20 (P.O.) | 10 (H.U.)<br>70 (K.A.)<br>20 (P.O.) | 10 (H.U.)<br>70 (K.A.)<br>20 (P.O.) | 30 (S.C.-c.)<br>70 (S.K.) | 30 (S.C.-c.)<br>70 (S.K.) |
| Rapid fermentation | 40 (K.A.)<br>60 (S.C.-c.) | 10 (C.L.)<br>10 (H.U.)<br>80 (S.C.-c.) | 10 (H.U.)<br>20 (M.P.)<br>20 (S.C.-s.)<br>50 (S.C.-c.) | 10 (K.A.)<br>10 (S.K.)<br>80 (S.C.-c.) | 90 (K.A.)<br>10 (S.C.-c.) | 10 (H.U.)<br>20 (S.C.-ch.)<br>70 (S.C.-c.) | 50 (K.A.)<br>50 (S.C.-ch.) | 60 (K.A.)<br>40 (S.C.-s.) | 10 (S.E.)<br>90 (S.C.-c.) |
| Malolactic fermentation | 20 (S.C.-s.)<br>80 (S.C.-c.) | 10 (K.A.)<br>10 (S.C.-s.)<br>80 (S.C.-c.) | 10 (S.C.-s.)<br>90 (S.C.-c.) | 10 (S.C.-s.)<br>10 (S.C.-ch.)<br>80 (S.C.-c.) | 80 (S.C.-b.)<br>20 (S.C.-c.) | 10 (K.A.)<br>60 (S.C.-b.)<br>10 (S.C.-c.)<br>20 (S.C.-a.) | 10 (K.A.)<br>20 (S.C.-s.)<br>60 (S.C.-c.)<br>10 (S.C.-p.) | 10 (K.A.)<br>60 (S.C.-s.)<br>30 (S.C.-c.) | 100 (S.C.-c.) |
| At bottling | 50 (S.C.-c.)<br>50 (S.C.-s.) | 40 (S.C.-s.)<br>60 (S.C.-c.) | 40 (K.A.)<br>20 (S.C.-s.)<br>40 (S.C.-c.) | 30 (C.P.)<br>60 (S.C.-c.)<br>10 (S.C.-s.) | 30 (C.P.)<br>70 (S.C.-c.) | 70 (C.P.)<br>30 (S.C.-c.) | 10 (H.A.)<br>30 (C.P.)<br>60 (S.C.-b.) | 90 (S.C.-c.)<br>10 (S.C.-s.) | 100 (S.C.-c.) |

[a] Abbreviations (Tables 3 and 4): B.N., *Brettanomices naardensis*; C.P., *Candida pelliculosa*; C.S., *Candida stellata*; C.U., *Candida utilis*; C.V., *Candida vartiovaarai*; C.Vi., *Candida vini*; C.L., *Cryptococcus laurentii*; H.A., *Hansenula anomala*; H.U., *Hanseniospora uvarum*; K.A., *Kloeckera apiculata*; M.P., *Metschnikowia pulcherrima*; P.M., *Pichia membranaefaciens*; P.O., *P. ohmeri*; S.C.-a., *S. cerevisiae*, race *aceti*; S.C.-b., *S. cerevisiae*, race *bayanus*; S.C.-c., *S. cerevisiae* race *cerevisiae*; S.C.-ca., *S. cerevisiae*, race *capensis*; S.C.-ch., *S. cerevisiae*, race *chevalieri*; S.C.-p., *S. cerevisiae*, race *prostoserdowii*; S.C.-s, *S. cerevisiae*, race *steinieri*; S.E., *S. exiguus*; S.K., *S. kluyveri*.

must (M1) samples (16), and the number of species was higher in must from unwashed apples than in that from washed apples (Table 4). This could be explained by the influence of surface strains, because with the traditional system a higher number of oxidative and low fermentative species were isolated from the fresh must. At the experimental plant, the most frequent yeast species isolated from fresh must were *Saccharomyces kluyveri*, *Kloeckera apiculata*, and *Saccharomyces cerevisiae*. In addition, we found *Hanseniaspora uvarum* (the perfect state of *K. apiculata*) and *Pichia ohmeri*. *S. kluyveri* was not found in the later stages of fermentation. *S. cerevisiae* concentrations increased throughout the following stages, and the highest level was found during the malolactic fermentation (90 to 100%). This pattern was similar for both technologies and for both apple mixtures. The races of *S. cerevisiae* isolated were *cerevisiae*, *steineri*, and *bayanus*, with a low concentration of *chevalieri*, *aceti*, and *prostoserdovii*. However, some authors have suggested that standard morphological and physiological tests appear incapable of discriminating all species (15).

The apiculate yeast *K. apiculata* was isolated in all the samples (during the rapid and malolactic fermentations in only very low concentration). In wines, this yeast is isolated during the early stages of fermentation and eventually dies off, leaving *S. cerevisiae* as the dominant species to complete the fermentation (6, 10), but in cider, this yeast remains through the later stages because of the low alcohol content of

TABLE 4. Yeast species identified during cidermaking in traditional Asturian cider plants

| Stage | Yeast percentage(s)[a] | | | | | |
|---|---|---|---|---|---|---|
| | Cider plant I | | Cider plant II | | Cider plant III | |
| | Barrel 1 | Barrel 2 | Barrel 1 | Barrel 2 | Barrel 1 | Barrel 2 |
| Must | 20 (C.V.)<br>30 (K.A.)<br>40 (S.C.-c.)<br>10 (S.C.-b.) | 10 (B.N.)<br>30 (K.A.)<br>30 (S.C.-c.)<br>30 (S.C.-b.) | 20 (C.Vi.)<br>10 (K.A.)<br>70 (S.C.-c.) | 30 (K.A.)<br>30 (S.C.-b.)<br>40 (S.C.-c.) | 10 (P.M.)<br>10 (K.A.)<br>10 (S.C.-b.)<br>70 (S.C.-c.) | 30 (K.A.)<br>30 (S.C.-b.)<br>40 (S.C.-c.) |
| Rapid fermentation | 10 (K.A.)<br>90 (S.C.-c.) | 20 (S.C.-b.)<br>80 (S.C.-c.) | 10 (C.Vi.)<br>10 (K.A.)<br>80 (S.C.-c.) | 20 (K.A.)<br>40 (S.C.-b.)<br>40 (S.C.-c.) | 100 (S.C.-c.) | 10 (C.U.)<br>10 (K.A.)<br>40 (S.C.-b.)<br>40 (S.C.-c.) |
| Malolactic fermentation | 100 (S.C.-c.) | 10 (S.C.-b.)<br>90 (S.C.-c.) | 100 (S.C.-c.) | 10 (K.A.)<br>90 (S.C.-c.) | 100 (S.C.-c.) | 100 (S.C.-c.) |
| At bottling | 10 (C.V.)<br>10 (K.A.)<br>10 (S.C.-c.)<br>20 (S.C.-ch.)<br>50 (S.C.-b.) | 10 (K.A.)<br>90 (S.C.-c.) | 20 (K.A.)<br>20 (S.C.-ca.)<br>60 (S.C.-c.) | 10 (C.S.)<br>10 (K.A.)<br>80 (S.C.-c.) | 10 (K.A.)<br>90 (S.C.-c.) | 100 (S.C.-c.) |

[a] Abbreviations of species are as defined in Table 3, footnote *a*.

the product. The oxidative species *Candida pelliculosa* isolated from bottles was probably a contaminant during bottling.

In Asturian cider plants, *S. cerevisiae* was isolated from fresh must in 50 to 80% concentrations. *K. apiculata* was found at all of the Asturian factories studied. In addition, *Candida vartiovaarai*, *Candida vini*, *Brettanomyces naardenensis*, and *Pichia membranaefaciens* were identified at this stage. The evolution of *S. cerevisiae* and *K. apiculata* were similar at the plant pilot. At bottling, we observed the presence of contaminant yeast species such as *C. vartiovaarai* and *Candida stellata*.

The most frequent races of *S. cerevisiae* isolated were *cerevisiae* and *bayanus*. The races *capensis* and *chevalieri* were found in low numbers (<1.0%). *S. cerevisiae* grows on the equipment (1, 5), which could explain why cider plants with poor hygienic conditions have a greater representation of this genus at the time of maximum fermentation (Tables 3 and 4) than our pilot plant with a stricter regimen.

Noticeably absent were *Saccharomycodes ludwigii*, which has been reported as an important spoilage yeast (3) and *Schizosaccharomyces pombe*, a maloalcoholic fermentation yeast (8, 19), both of which have not been detected in Asturian cider.

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## LITERATURE CITED


1. **Beech, F. W.** 1972. Cider making and cider research: a review. J. Inst. Brew. **78**:477–491.
2. **Beech, F. W., and J. G. Carr.** 1960. Selective media for yeasts and bacteria in apple juice and cider. J. Sci. Food Agric. **11**:38–40.
3. **Beech, F. W., and J. G. Carr.** 1977. Cider and perry, p. 179–183. *In* A. H. Rose (ed.), Economic microbiology, vol. 1. Academic Press, Inc. (London), Ltd., London.
4. **Beech, F. W., and S. W. Challinor.** 1951. Report of the Agricultural and Horticultural Research Station at the Res. Stn University of Bristol for 1950. University of Bristol, Bristol, England.
5. **Beech, F. W., and R. R. Davenport.** 1970. The role of yeast in cidermaking, p. 73–146. *In* A. H. Rose and I. S. Harrison (ed.), Yeasts, vol. 3. Academic Press, Inc. (London), Ltd., London.
6. **Fleet, G. H., S. Lafon-Lafourcade, and P. Riberau-Gayon.** 1984. Evolution of yeast and lactic acid bacteria during fermentation and storage of Bordeaux wines. Appl. Environ. Microbiol. **48**:1034–1038.
7. **Fornachon, J. C. M.** 1968. Influence of different yeasts on the growth of lactic acid bacteria in wine. J. Sci. Food Agric. **19**:373–378.
8. **Fournier, H., R. Charbonneau, M. Gagnon, and G. Dubois.** 1981. L'effect desacidificant de *Schizosaccharomyces pombe* sur le cidre du Quebec. Sci. Aliment. **1**:19–25.
9. **Greenhouse, S. W., and S. Geiser.** 1959. Methods in the analysis of profile data. Psychometrika **32**:95–112.
10. **Heard, G. M., and G. H. Fleet.** 1985. Growth of natural yeast flora during the fermentation of inoculated wines. Appl. Environ. Microbiol. **50**:727–728.
11. **Kreger-Van Rij, N. J. W.** 1984. Yeasts: a taxonomic study. Elsevier Science Publisher, B.V., Amsterdam.
12. **Lemaresquier, H.** 1987. Interactions levures-bacterias. Rev. Fr. Oenologie **109**:57–60.
13. **Litell, R. C.** 1989. Statistical analysis of experiments with repeated measurements. Hortic. Sci. (Stuttgart) **24**:37–42.
14. **Mafart, P.** 1986. Influence de la flore de fermentation sur la flaveur des cidres et selection des souches. Bios **17**:33–37.
15. **Martini, A. V., and C. P. Kurtzman.** 1985. Deoxyribonucleic acid relatedness among species the genus *Saccharomyces* sensu stricto. Int. J. Syst. Bacteriol. **35**:508–511.
16. **Michel, A., C. Bizeau, and J. F. Drilleau.** 1988. Flore levurienne presente dans les cidreries de l'ouest de la france. Sci. Aliment. **8**:359–368.
17. **Poulard, A., R. Reberteau, and Y. Rousset.** 1985. La microflore des phases prefermentaires. Rev. Fr. Oenologie **99**:14–19.
18. **Rowell, J. G., and D. E. Walters.** 1976. Analysing data with repeated observations on each experimental unit. J. Agric. Sci. **87**:423–432.
19. **Taillandier, P., and J. P. Riba.** 1988. Malate utilization by *Schizosaccharomyces pombe*. Biotechnol. Lett. **10**:469–472.
20. **Whiting, C. G.** 1973. Acetification in ciders and perries. J. Inst. Brew. **79**:218–226.