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CONTROLLED PRODUCTION OF CIDER BY INDUCTION OF ALCOHOLIC FERMENTATION AND MALOLACTIC CONVERSION

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Characterization experiments involving lactic bacteria allowed a strain of *Leuconostoc oenos* to be selected in terms of growth capacity at variable pH, temperature, ethanol and sulphite concentrations, malic to lactic conversion yield, acetic acid uptake and dextran production capacity.

Cider was produced under controlled conditions where the effect of *Kloeckera apiculata* yeasts on the development of *Saccharomyces cerevisiae* and production of major non-volatile compounds influencing end product quality was studied. The apiculate yeast was found to produce large amounts of acetic acid and use the other organic acids; also, it hindered fermentation to a certain extent. A study of the effect of the inoculation time with *L. oenos* as an inducer of malolactic fermentation revealed sequential inoculation of the lactic bacterium once most major sugars in the must had been depleted to be the most favourable. Using yeast cell walls boosted fermentation development, as well as degradation of malic acid and synthesis of succinic and acetic acid.

Key Words: *Saccharomyces cerevisiae, Kloeckera apiculata, Leuconostoc oenos, yeast cell walls, cider.*

INTRODUCTION

Alcoholic fermentation and malolactic conversion are two microbiological processes of a great significance to cider production in Asturias (Northern Spain). Alcoholic fermentation proceeds rapidly and usually depletes most sugars in the must within 1–3 weeks. In traditional cider making processes, fermentation is effected by wild yeasts from apples and cellar utensils. Alternatively, selected cultures of *Saccharomyces cerevisiae*, which give rise to a high randomness in the quality of the resulting cider, have lately started to be used. Some authors claim that fermentations relying exclusively on this yeast species are of a lower quality than those resulting from the combination of an apiculate and a fermentation yeast[13]. Based on previous taxonomic studies performed by the authors' group[7], the most appropriate yeast combination for production of Asturian cider is that of *Kloeckera apiculata* and *Saccharomyces cerevisiae*. Some authors have reported these two species to have a mutual boosting effect on growth[21].

As regards malolactic fermentation, the chemical composition of cider occasionally hinders growth of malolactic yeasts. The low pH, the potential presence of sulphur dioxide, the ethanol concentration and the scarcity of nutrients in the medium, in addition to the low temperatures where cider is usually made in Asturias, are the most significant inhibitory factors in this respect.

Inoculation with *Leuconostoc oenos* for induction of malolactic fermentation has opened up new avenues for development of new cider making procedures.

During alcoholic fermentation, a large number of by-products result from yeast metabolism including fatty acids of medium chain length that inhibit yeast growth and slow down or even stop fermentation altogether[30]. A spontaneous end of must fermentation before sugars are fully converted poses serious problems to the cider making industry; the cider does not reach the desired alcoholic strength and residual sugars make an excellent source for development of undesirable bacterial; this can be countered by using yeast cell walls during fermentation[31].

A deeper knowledge of the interactions of strong and weak fermentation yeasts with lactic bacteria may allow one to induce alcoholic fermentation and malolactic fermentation in cider in a rapid, reproducible manner and hence to raise the quality of the end product. To this end, it is necessary to establish the optimum conditions for development of mixed yeast–lactic bacterium cultures[3].

In this work, controlled laboratory fermentations were carried out using fresh apple must which was pre-sterilized by filtration in order to investigate the effect of *Kloeckera apiculata* and the inoculation time of *Leuconostoc oenos*, the fermentation temperature and the addition of yeast cell walls on the ecological equilibrium of the cider and its non-volatile components.

EXPERIMENTAL METHODS

Materials

The must used was obtained from a mixture of apples of variable sensory properties that are traditionally used by the Asturian cider making industry: 30% Collaos (slightly sour), 40% Raxao (sour), 10% Durón Arroes (sweet), 15% Coloradona (sweet-bitter) and 5% Meana (bitter-sour). The must was extracted by means of a grating mill and a double-tray hydraulic press, and was stored at −20°C. Prior to use, the must was microfiltered through a microporous tangential device using a polyether sulphone plate of 0.33 µm mesh, and subsequently sterilized by passage through a Durapore filter of 0.2 µm pore size.

Experimental conditions

Fermentations were carried out in pre-sterilized 2 l jacketed reaction flasks that were shaken for 15 min every 2 h. During the first 24 h, inocula were allowed to grow aerobically in order to favour cell replication; subsequently, a nitrogen stream was passed in order to maintain anaerobic conditions. A volume of 2 l of apple must was inoculated with the yeast at a final concentration of 10^6 cells/ml. Fermentations were conducted at 12 and 18°C.

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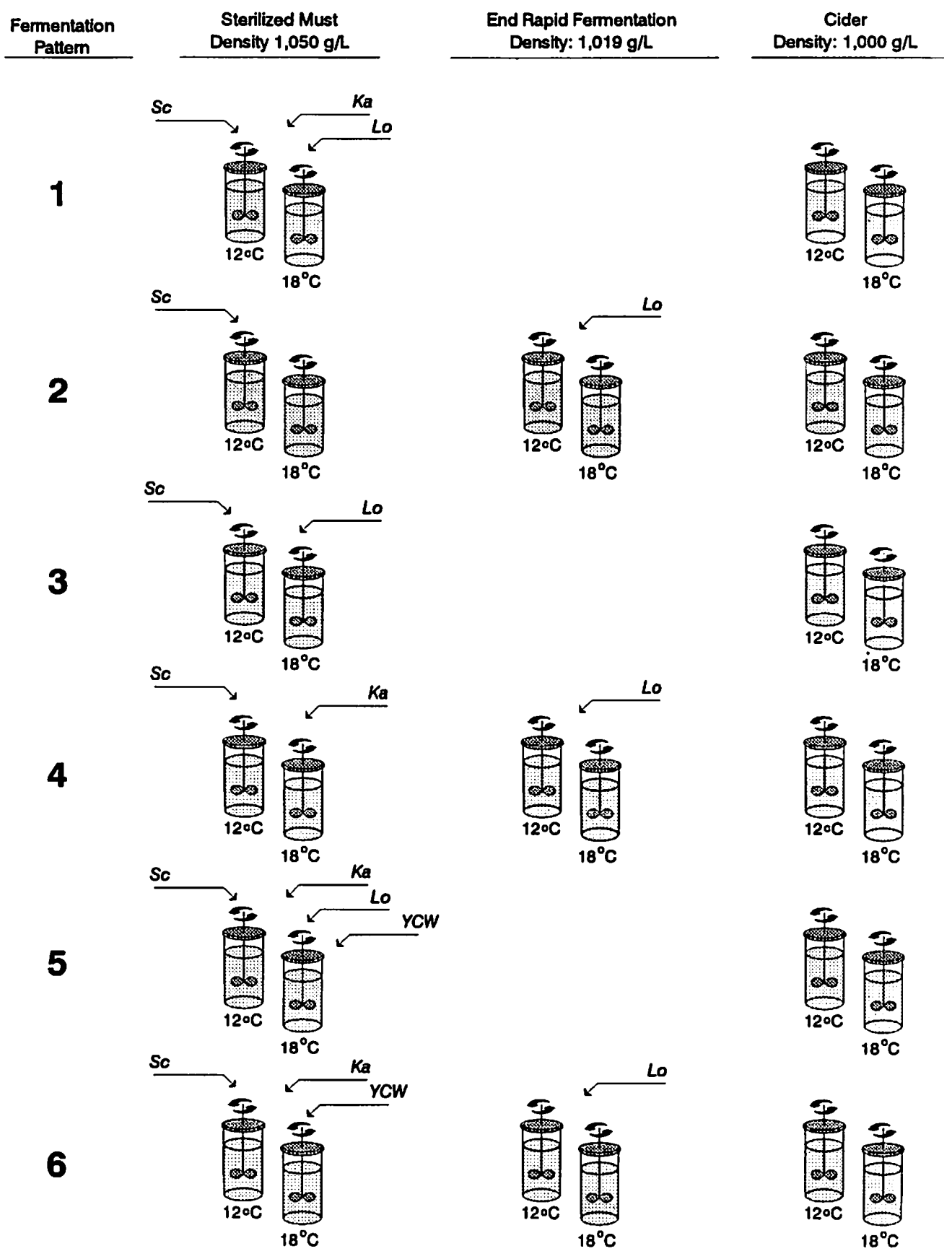

FIG. 1. Experimental set-up used.

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The effect of the presence of the apiculate yeast and the best time for inoculation with the lactic bacterium were determined by using the experimental set-up depicted in Fig. 1.

In those tests involving the three types of inocula, the effect of adding yeast cell walls (YCW) on the fermentation process was investigated. All tests were carried out in triplicate.

Bacteria and yeast strains

The *S. cerevisiae* and *K. apiculata* strains used belonged to the authors' laboratory collection. The strains were previously isolated from spontaneous industrial cider fermentation media, taxonomically identified and physiologically and biochemically characterized. The *L. oenos* strain was isolated and identified in previous work.

Cell walls

The cell wall concentration used in the tests was 400 ppm. The additive, supplied by Carburos Metálicos SA (Asturias, Spain), was added 48 h after yeast inoculation.

Microbiological counting

The populations of the different microorganisms involved were monitored by analysing samples from the fermenting musts taken every 48 h until a density of 1,000 g/l was reached. Serial 1:10 dilutions were performed with Ringer serum, and the following selective media were used for quantitative analyses: (a) MRS supplemented with the antifungal antibiotic pimaricin at a 25 ppm concentration (surface seedings were conducted in duplicate and dishes were incubated in anaerobosis jars under an H_2–CO_2 atmosphere at 28°C for 5 days); (b) YMA containing 25 ppm penicillin and 100 ppm streptomycin (to ensure inhibition of lactic and acetic bacterial growth) for total yeast counting (seeding was also carried out superficially and incubation was performed at 28°C for 48 h); (c) Lysine agar[23], obtained from Oxoid (Barcelona, Spain), for *K. apiculata* (incubation lasted 24 h at 28°C); and (d) selective ethanol–sulphite–agar medium[14], an inhibitor for wild yeast growth, for *S. cerevisiae* counting (dishes were incubated at the same temperature as the other cultures for 48 h).

Analytical methods

Sugars (glucose, fructose, sucrose), ethanol and polyols (glycerol and sorbitol) were determined by ion-exclusion HPLC using the method of Blanco *et al.*[5]. Samples were extracted by passage through Sep-Pak C_{18} cartridges and microfiltration through 0.22 µm mesh. The chromatographic column used was a Sugar Pak I model (300 × 6.5 mm i.d.). The operating conditions were as follows: mobile phase 50 ppm $Ca(Na)_2EDTA$, $q = 0.5$ ml/min; column temperature 80°C; detector temperature 37°C; detector sensitivity 32; injected volume 10 µl.

Major organic acids (shikimic, malic, lactic, acetic and succinic) were quantified by reversed-phase HPLC using the method of Blanco *et al.*[6]. D-lactic acid was enzymatically determined according to Bergmeyer[4]. L-lactic acid was determined by difference between total lactic acid and D-lactic acid. Samples were microfiltered through 0.22 µm mesh. A 250 × 4 mm i.d. Spherisorb ODS column and the following chromatographic conditions were used; mobile phase 0.01 M KH_2PO_4/H_3PO_4, pH 2.25, $q = 0.5$ ml/min; column temperature 25°C; injected volume 20 µl. All acids were monitored at $\lambda = 206$ nm. Total and volatile acidity were determined using AOAC's recommended methods. Total nitrogen was quantified by using a modified version of the Hach *et al.*[12] method. Finally, phenol compounds were determined according to Montreau[22].

Statistical analysis

The results obtained were subjected to a factor variance analysis using the SAS[28] statistical package. The following variables were analysed: presence of *K. apiculata*, inoculation time of *L. oenos*, temperature and addition of yeast cell walls. Tukey's HSD Test was also employed.

Results and Discussion

Selection of the Leuconostoc oenos

The need to choose an appropriate autochthonous lactic bacterial strain to boost and drive malolactic fermentation has been widely demonstrated[11,24].

The lactic bacteria used in this work was chosen on the basis of the following criteria: the ability to grow at different ethanol concentrations, pH values, SO_2 concentrations and temperatures; the malolactic conversion rate; the ability to accumulate acetic acid; and the dextran producing activity (Table I). Tolerance to ethanol and a low pH value are two of the most crucial properties for selecting lactic bacteria for inducing malolactic fermentation in alcohol drinks[16].

The tolerance to ethanol of 10 randomly chosen strains from those previously identified at our pilot cellar was studied. All exhibited a similar response to the concentrations tested (Table I), which ranged from 5 to 11%; exceptionally, growth of strains IC11 and IC12 in the liquid medium was inhibited at a 9% ethanol concentration.

As regards tolerance to SO_2 (added as potassium metabisulphite), most of the strains (IC98, IC99 and IC910 excluded) exhibited slightly inhibited growth at 125 ppm. Strains IC11 and IC96 stopped growing above 150 ppm and only IC13 continued to grow above a potassium metabisulphite concentration of 175 ppm. The usual practice among cider makers of using this additive to prevent or stop proliferation of undesirable microorganisms (e.g. acetic bacteria or even some species of lactic bacteria) entails selecting an SO_2 resistant strain to induce malolactic fermentation, as previously noted for wines by Fornachon[9].

Growth of all the lactic bacteria tested in the liquid medium started to decline below pH 3.25; growth was very weak at pH 3.00 and stopped altogether by pH 2.75. Because the usual pH during the cider making progress is 3.50–3.75, none of the strains studied is bound to be affected as regards growth in the liquid medium.

One other major factor to consider in selecting a lactic bacterial strain is its ability to grow at low temperatures. Most of the strains studied were found to grow unrestrictedly at 18°C; however, culture turbidity decreased markedly on lowering the temperature to 12°C.

Finally, the ability of *L. oenos* strains to convert malic acid into lactic acid was studied. Strains IC11 and IC910 exhibited the highest production of L-lactic acid and the least liability to accumulate acetic acid. This test, which is essential for appropriate selection of the lactic bacteria, resulted in the greatest differences between the strains studied. Also, as can be seen in Table I, none of the strains exhibited dextran producing activity.

In view of the results obtained, strains IC11 and IC910, with similar technological features, were short-listed. IC11 was finally adopted because it exhibited the lower residual concentration of malic acid.

Microorganism growth

The variance analysis aimed at detecting potential differences between changes in the yeast and bacterial populations as a function of the fermentation system used was carried out at three significant stages of the fermentation process, namely: during tumultuous fermentation, during malolactic fermentation and at the end of fermentation.

From the statistical analysis it follows that the presence of *K. apiculata* in the reactor had a significant effect ($p = 0.0120$) on the *S. cerevisiae* population during tumultuous fermentation; the counts of the latter were lower when the apiculate yeast

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TABLE I Selection tests for *Leuconostoc oenos*

		IC11	IC12	IC13	IC14	IC15	IC96	IC97	IC98	IC99	IC910
Tolerance to ethanol	5%	+	++	++	++	++	++	++	++	++	++
	7%	++	++	++	++	++	++	++	++	++	++
	9%	++	+	++	++	++	++	++	++	++	++
	11%	+	+	++	++	++	++	++	++	++	++
Tolerance to potassium metabisulphite	75 ppm	++	++	++	++	++	++	++	++	++	++
	100 ppm	+	+	+	+	+	+	+	+	+	+
	125 ppm	d	d	d	d	d	d	d	+	+	+
	150 ppm	–	d	d	d	d	–	d	d	d	d
	175 ppm	–	–	d	–	–	–	–	–	–	–
Dextran		–	–	–	–	–	–	–	–	–	–
Tolerance to a low pH	2.75	–	–	–	–	–	–	–	–	–	–
	3.00	d	d	d	d	d	d	d	d	d	d
	3.25	+	+	+	+	+	+	+	+	+	+
	3.50	++	++	++	++	++	++	++	++	++	++
L-malic (g/l)	5.84*	0.05	0.23	0.19	1.39	1.43	1.45	0.00	0.01	0.05	0.26
L-Lactic (g/l)	0.05*	3.05	2.87	2.87	2.31	2.28	2.24	2.47	2.49	2.03	2.95
Acetic (g/l)	0.00*	6.83	7.06	7.06	12.39	11.78	11.32	12.94	12.56	10.47	6.55
Growth	12°C	d	d	d	+	d	d	d	d	d	d
	18°C	d	d	d	++	++	++	++	++	+	+

– absence of growth, + abundant, ++ highly abundant, *initial amount.

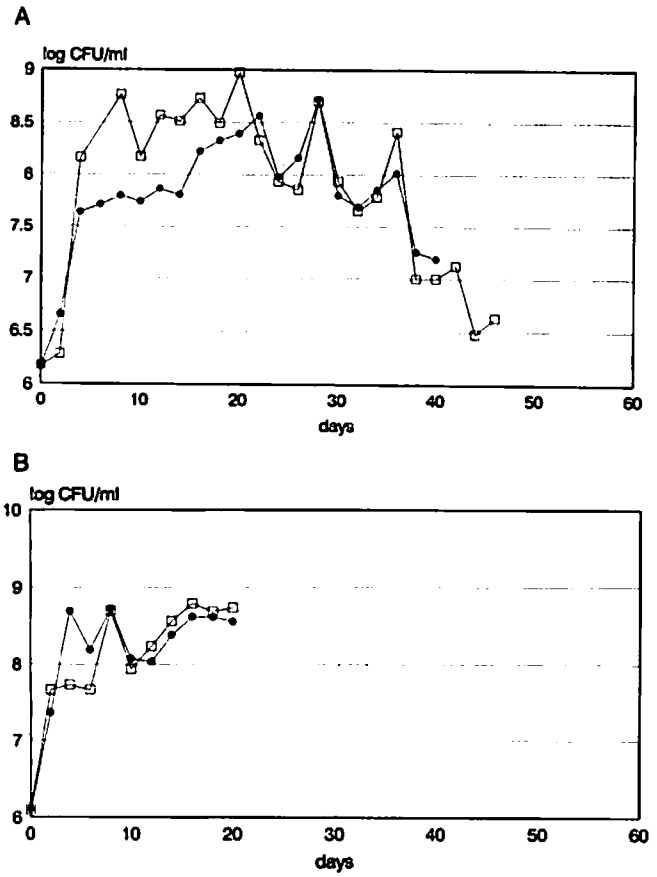

FIG. 2. Influence of the presence of *K. apiculata* on changes in the population of *S. cerevisiae* at 12°C (A) and 18°C (B) (□) Type 3. (•) Type 1.

was inoculated simultaneously and a low fermentation temperature was used (Fig. 2). On the other hand, the time *L. oenos* was inoculated had no effect on cell replication in *S. cerevisiae* at this stage. Mutual growth interactions between *L oenos* and *S. cerevisiae* should be taken into account in developing a microorganism inoculum model[14].

During malolactic fermentation, the *S. cerevisiae* population was significantly influenced by the presence of *K. apiculata* ($p = 0.0019$) and the temperature ($p = 0.0039$); counts increased with increasing temperature and the absence of the yeast during the first stage (Fig. 2). The addition of yeast cell walls had no significant effect on changes in the fermentation yeast. The toxic effect of secreted fatty acids of medium-length chain on the yeast itself are difficult to predict, so estimating the effect of adding cell walls on the fermentation yeast growth is a difficult task, as shown by Lonvaud-Funel *et al.*[19].

The *K. apiculata* population was significantly affected by the inoculation time ($p = 0.0976$) and fermentation temperature ($p = 0.006$) at all the stages considered (Fig. 3). It should be noted that the counts for this species were higher when the lactic bacteria was inoculated sequentially and no survival factors were added. Low temperatures, which boost growth of weak fermentation species, result in decreased ethanol concentrations in the end product and in increased amounts of residual sugars that can be metabolized via secondary pathways with dubious effects on product quality. As can be seen in Fig. 3, *K. apiculata* counts were influenced by the temperature, especially when *L. oenos* was simultaneously inoculated—the cell density detected at 18°C was lower in this case.

Malolactic fermentation was significantly affected by the temperature ($p = 0.0037$), the inoculation time ($p = 0.0876$), its interaction with the presence of *K. apiculata* ($p = 0.0876$) and that with the previous three factors ($p = 0.0513$). Counts of the lactic bacteria were higher at 18°C and in the fermentations where inoculation was simultaneous (Fig. 4). At 12°C cell replication in lactic bacteria is considerably slowed down and malolactic fermentation rate was slower.

The temperature proved highly influential on the results; previously Gao and Fleet[10] had studied the effect of *Saccharomyces* strains and the so-called "first-stage yeasts" on wines. In practice, inoculating mixed cultures consisting of an apiculate species and a fermentation yeast on non-sterile must can substantially complicate fermentation control[25].

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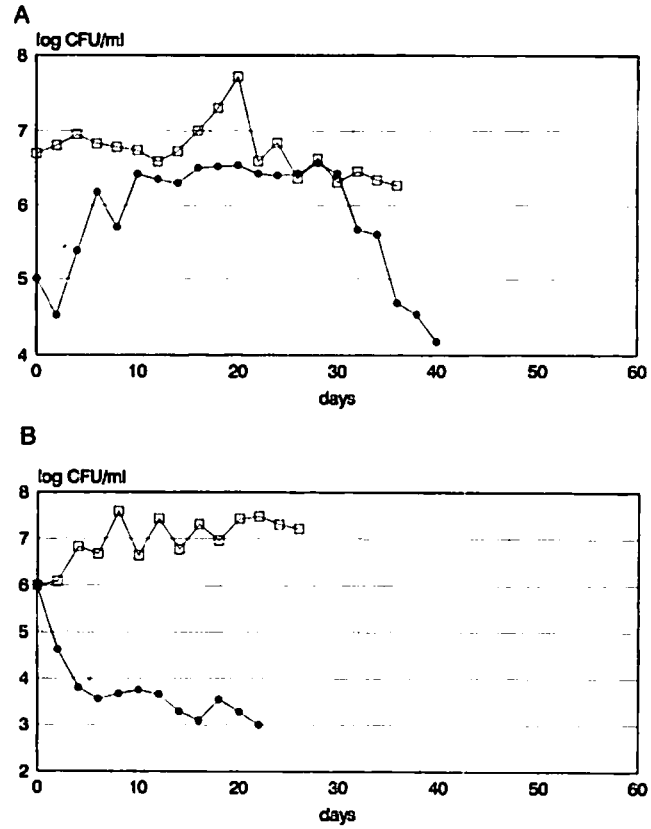

FIG. 3. Influence of the time of inoculation with the lactic bacteria on changes in the population of *K. apiculata* at 12°C (A) and 18°C (B). (□) Type 4. (•) Type 1.

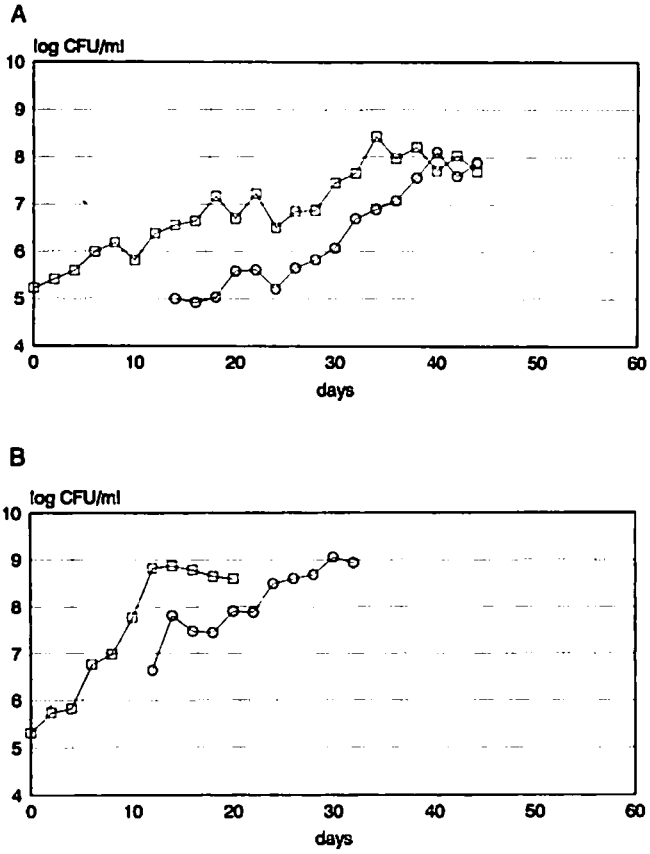

FIG. 4. Influence of the time of inoculation with the lactic bacteria on changes in the population of *L. oenos* at 12°C (A) and 18°C (B). (□) Type 3. (○) Type 2.

Sugars and alcohols

The uptake of major sugars (fructose, glucose and sucrose) by apple must during fermentation was found to be independent of the variables studied. It should be noted that these compounds were virtually completely metabolized in the 36 experimental units (Table II). Glucose was particularly prominent in this respect, with a zero concentration in all tests—this is logical taking into account that it was the most readily metabolized carbohydrate for the microorganisms.

Glycerol production was not affected by the experimental variables. The greatest accumulation of ethanol was observed in the fermentation at 18°C ($p = 0.0009$) where *L. oenos* was inoculated during the slow fermentation stage ($p = 0.0693$), *K. apiculata* was absent from the starting must ($p = 0.0590$) and the addition of yeast cell walls boosted ethanol production ($p = 0.0440$). As noted earlier, the highest cell density for *S. cerevisiae* was measured at 18°C in the absence of *K. apiculata*, which is consistent with the increased ethanol content obtained under such conditions.

The presence of *K. apiculata* resulted in significant uptake ($p = 0.0721$) of the major polyol in apples: sorbitol. The final concentration of this sugar was slightly higher at 18°C ($p = 0.0035$) than it was at 12°C, and when the lactic bacteria was inoculated in a sequential manner. On the other hand, the presence of yeast cell walls significantly increased the final concentration of this polyol ($p = 0.0693$).

Organic acids

Malic acid initially present in the must was fully metabolized in all tests (Table III); a significant interaction ($p = 0.0315$) between the effects of temperature and the addition of yeast cell walls was observed. However, the highest L-lactic concentration was obtained when no cell walls were added ($p = 0.0107$), which is consistent with the increased cell density of *L. oenos* in fermentations excluding this additive. In this respect, it should be borne in mind that not all lactic acid produced in fermentations comes from malolactic fermentation. Also, the presence of *K. apiculata* was found to significantly influence ($p = 0.0114$) accumulation of the acid; in fact, the amount of lactic acid obtained was greater in those fermentations where the yeast was absent.

Changes in the shikimic acid concentration during fermentation were influenced by the experimental variables. Accumulation of this acid was lower in the presence of the apiculate yeast ($p = 0.0009$) at 12°C ($p = 0.0012$) when the lactic bacteria was inoculated during the slow fermentation ($p = 0.0007$).

The amount of acetic acid accumulated at the end of fermentation was not too large (the maximum concentration was 0.29 g/l); it was affected by the addition of cell walls ($p = 0.0391$) and its interaction with the inoculation time ($p = 0.0004$). It should be noted that the addition of cell walls and the presence of the weak fermentation yeasts, in combination with a simultaneous inoculation of the lactic bacteria ($p = 0.0078$), resulted in the highest acetic acid concentration, which can be ascribed to the high acetifying capacity of *K. apiculata* and *L. oenos* in the presence of a non-limiting concentration of carbohydrates and other factors boosting its metabolism (e.g. yeast cell walls). Such a high acetifying capacity of *K. apiculata* was previously noted by other authors[26] as one of its major disadvantages for controlled use in alcoholic beverage production.

D-lactic acid is linked to carbohydrate metabolism in yeasts and lactic bacteria. It was found that production of this acid was higher in the absence of yeast cell walls ($p = 0.0003$). The heterogeneous fermentation metabolism of *L. oenos* produces acetic and D-lactic acid simultaneously, which accounts for the more extensive growth of this microorganism in the absence of cell walls.

The presence of *K. apiculata* decreased the production of succinic acid ($p = 0.0003$); the interactions of this factor with the type of lactic bacterial inoculation ($p = 0.0565$) and with

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TABLE II Sugar and alcohol concentrations in must and cider

		Sucrose (g/l)		Glucose (g/l)		Fructose (g/l)		Glycerol (g/l)		Ethanol (g/l)		Sorbitol (g/l)	
Type	°C	x	σ	x	σ	x	σ	x	σ	x	σ	x	σ
Type 1	12	ND	–	ND	–	ND	–	4.55	0.29	50.87	0.86	8.14	0.07
	18	ND	–	ND	–	0.14	0.25	3.27	1.68	31.27	19.05	5.33	3.18
Type 2	12	ND	–	ND	–	ND	–	4.73	0.45	50.72	1.22	8.09	0.32
	18	ND	–	ND	–	ND	–	4.51	1.18	57.05	2.73	9.62	0.68
Type 3	12	ND	–	ND	–	ND	–	4.20	0.64	44.79	7.43	7.06	1.15
	18	ND	–	ND	–	ND	–	4.98	0.25	49.88	6.94	8.59	0.67
Type 4	12	0.08	0.13	ND	–	ND	–	3.93	0.60	42.02	4.65	6.58	1.05
	18	ND	–	ND	–	ND	–	9.06	4.89	52.10	0.79	8.05	0.65
Type 5	12	ND	–	ND	–	ND	–	5.01	0.52	50.99	4.00	8.15	0.59
	18	ND	–	ND	–	ND	–	5.61	0.91	53.34	3.99	8.33	0.39
Type 6	12	ND	–	ND	–	ND	–	5.23	0.57	45.59	3.85	7.11	0.83
	18	ND	–	ND	–	ND	–	5.33	0.43	52.73	2.08	8.56	0.38
MUST		24.03		19.71		60.31		0.00		0.00		7.76	

ND not detected
x mean, σ standard deviation

TABLE III Organic acid concentrations in must and cider

		Malic (g/l)		Shikimic (ppm)		Acetic (g/l)		Succinic (g/l)		D-Lactic (g/l)		L-Lactic (g/l)	
Type	°C	x	σ	x	σ	x	σ	x	σ	x	σ	x	σ
Type 1	12	0.02	0.01	28.11	4.81	0.14	0.06	2.06	0.09	0.39	0.05	3.35	0.76
	18	0.01	0.01	16.11	7.48	0.13	0.02	1.44	0.66	0.42	0.12	3.71	1.05
Type 2	12	0.06	0.06	30.80	2.81	0.09	0.08	2.72	0.03	0.40	0.04	4.63	0.34
	18	0.19	0.31	29.03	5.30	0.16	0.09	3.45	0.57	0.37	0.02	3.92	0.06
Type 3	12	0.02	0.01	25.36	3.40	0.22	0.09	2.51	0.36	0.41	0.08	4.10	0.27
	18	0.04	0.02	29.84	4.39	0.16	0.07	2.21	1.29	0.39	0.06	3.68	0.50
Type 4	12	0.03	0.02	15.06	2.60	0.29	0.05	1.49	0.18	0.32	0.03	3.59	0.03
	18	0.01	0.01	26.71	0.52	0.22	0.09	1.53	0.14	0.34	0.07	3.06	0.64
Type 5	12	0.01	0.01	15.81	1.39	0.25	0.05	1.39	0.08	0.37	0.03	3.31	0.36
	18	0.01	0.01	21.90	3.00	0.27	0.00	1.73	0.24	0.20	0.02	2.50	0.26
Type 6	12	0.01	0.01	15.63	1.45	0.20	0.02	1.31	0.15	0.25	0.02	2.70	0.26
	18	0.01	0.01	23.93	2.08	0.22	0.02	1.87	0.10	0.24	0.02	2.65	0.26
MUST		3.59		16.72		0.00		0.00		0.00		0.00	

x mean, σ standard deviation

the fermentation temperature ($p = 0.0042$) were also significant. As a result of the close relationship of sugar and amino acid metabolisms (notably that for glutamic acid) with the synthesis of succinic acid, the combination of sequential inoculation with the lactic bacteria and the exclusive presence in the must of the fermentation yeast *S. cerevisiae* resulted in increased accumulation of the acid. The enhancing effects of temperature and the absence of *K. apiculata* on the metabolism of fermentation yeasts is consistent with the results. The amount of succinic acid measured in the absence of the apiculate yeast in the fermentations at 18°C exceeded all others.

Overall parameters

Total acidity was found to be influenced both by the presence of *K. apiculata* in the reactor ($p = 0.0001$) and by the temperature ($p = 0.0426$). The highest total acidity was measured in types 2 and 3 (3.75–4.29 g/l), from which the apiculate yeast was excluded. These results may be related to the ability of this species to use some organic acids. The addition of yeast cell walls had a significant effect on total acidity ($p = 0.0185$), which was higher in their absence. Low concentrations of ethanol and low pH, for example, 6% (vol/vol) ethanol and pH 3 (usual in cider) did not allow the solubilization of toxic acids such as octanoic acid and decanoic acid, and under those conditions they have no detectable toxic effect[27,18] over the growth of *S. cerevisiae*, contributing to an increase in the ethanol productivity of alcoholic fermentation[20].

Volatile acidity was influenced by the fermentation temperature ($p = 0.0005$): it was higher at 12°C, which is consistent with the increased capacity of *K. apiculata* to proliferate and produce acetic acid. Volatile acidity was also significantly affected by the addition of yeast cell walls ($p = 0.0001$) and its interaction with the other factors studied ($p = 0.0001$); and the presence of cell walls in the reactor increased volatile acidity.

Free nitrogen was affected by the following variables: the microorganism combination ($p = 0.0016$), temperature ($p = 0.0001$) and the interaction between both factors ($p = 0.0103$). Types 5 and 6, where yeast cell walls were used, exhibited higher final concentrations of free nitrogen (43.27–47.66 ppm).

The growth of apiculate yeasts is influenced by different fermentation factors, such as temperature and pH, and is affected by ethanol concentrations above 5–6%[16] and by sulfite[2]. It has been reported that initial profileration of apiculate yeasts is reflected in the sensory quality of fermented products[8]. Strains of these yeasts are known to produce concentrations of acetic acid, esters and glycerol higher than those produced by *S. cerevisiae*[17]. In contrast to the general

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TABLE IV Overall parameter values for must and cider

		pH		Total ac. (g/l)		Volatile ac. (g/l)		Nitrogen (ppm)		Polyphenols (g/l)		Density (g/l)	
Type	°C	x	σ	x	σ	x	σ	x	σ	x	σ	x	σ
Type 1	12	3.98	0.04	3.14	0.14	0.32	0.02	25.23	0.44	0.78	0.01	999.0	0.5
	18	3.53	0.10	3.06	0.75	0.20	0.17	32.46	8.87	0.55	0.32	998.0	1.0
Type 2	12	3.94	0.10	3.75	0.19	0.20	0.12	28.72	6.90	0.76	0.03	1000.6	0.5
	18	3.54	0.06	4.29	0.56	0.35	0.79	43.10	4.51	0.86	0.04	999.0	1.7
Type 3	12	3.90	0.06	3.75	0.19	0.32	0.02	29.97	7.70	0.73	0.06	999.6	1.5
	18	3.61	0.01	3.96	0.25	0.35	0.96	40.21	5.66	0.81	0.03	998.3	0.5
Type 4	12	3.72	0.08	2.36	0.08	0.40	0.03	24.56	3.22	0.75	0.02	999.0	0.5
	18	3.67	0.01	3.02	0.19	0.29	0.04	29.23	2.23	0.77	0.04	999.0	0.5
Type 5	12	3.80	0.01	2.28	0.07	0.41	0.06	24.61	4.26	0.83	0.12	999.0	0.5
	18	3.68	0.01	2.94	0.25	0.31	0.02	47.66	1.25	0.75	0.04	999.3	0.5
Type 6	12	3.72	0.06	2.28	0.07	0.42	0.08	43.27	9.83	0.77	0.01	999.0	0.5
	18	3.68	0.01	2.81	0.13	0.31	0.01	39.89	2.06	0.75	0.01	999.6	0.5
MUST		3.27		3.06		0.10		45.58		0.67		1050	

x mean, σ standard deviation

assumption, Romano *et al.*[26] found that *K apiculata* produced less than 1 g of acetic acid per litre and showed a low general production of higher alcohols in synthetic medium. The results reveal that the presence of *K. apiculata* in cider fermentation is in principle of little technological interest; in fact, it increases the concentration of unwanted products such as acetic acid and hinders growth of the fermentation yeast to some extent. In addition, the best way of inducing malolactic fermentation was found to be sequential inoculation of a selected strain of *L. oenos* once most sugars initially present in the must had been consumed. On the other hand, the presence of cell walls boosts alcoholic fermentation, malic acid degradation and the synthesis of succinic and acetic acid. These results are in accord with previous studies[29].

Finally a low fermentation temperature (below 12°C) favours development of weak fermentation yeasts such as *K. apiculata*, which hinders growth of *S. cerevisiae*, and development of alcoholic fermentation while slowing down growth of *L. oenos* and hence development of malolactic fermentation.

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