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Assessment of free and immobilized kefir culture in simultaneous alcoholic and malolactic cider fermentations

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2-methyl-1-propanol (isobutanol) (PubChem CID: 6560)
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ABSTRACT

The aim of the present study was to assess application of free or immobilized kefir culture on apple pieces and delignified cellulosic material (DCM) in simultaneous alcoholic and malolactic cider fermentations at a wide temperature range (5–45 °C). Repeated batch fermentations were continued for higher than 7 months, showing a high operational stability of the systems and were completed in less than 24 h with immobilized cells on DCM at 37 °C. Malic acid conversion up to 71.5% and ethanol productivity values up to 56.9 g/(Ld) were recorded, which could be adopted by the industrial sector. PCR-DGGE analysis of kefir culture showed no changes in microbial diversity during cell immobilization and fermentations. Analysis of major volatiles documented a significant increase of ethyl acetate content and a decrease of higher alcohols at low temperatures. HS-SPME GC/MS analysis revealed that the highest content of esters and total volatiles was observed in cider fermented by kefir culture immobilized on apple pieces at 20 °C. Principal Component Analysis showed that mainly the immobilization support rather than the temperature had a significant effect on volatile composition. Finally, the sensory evaluation ascertained the high quality of the new products.

1. Introduction

Cider-making is a complex process requiring both alcoholic and malolactic (ML) fermentation. During cider production, reduction of beverage acidity, microbial stability, and organoleptic improvement induced by ML fermentation are generally recognized as important phases for quality development. Therefore, attempts have been focused on simultaneous alcoholic and ML fermentation using mixed cultures consisting of yeasts and ML bacteria (Kourkoutas, Manolović, & Nedović, 2010).

Cell immobilization offers numerous advantages, such as enhanced fermentation productivity, ability for cell recycling, application of continuous configurations, enhanced cell stability and viability, and improvement of quality (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). In this vein, a few attempts

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have been carried out aiming at co-immobilization of *Saccharomyces cerevisiae* and ML bacteria (Nedovic et al., 2000; Servetas et al., 2013). Similarly, apple pieces and delignified cellulosic material (DCM) have been successfully tested as immobilization supports of yeasts and lactic acid bacteria for wine alcoholic and ML fermentations (Agouridis, Kopsahelis, Plessas, Koutinas, & Kanellaki, 2008; Kourkoutas, Komaitis, Koutinas, & Kanellaki, 2001; Koutinas et al., 2012).

Kefir is a consortium of microbes that is mainly used in the production of the low alcoholic, traditional Russian drink "kefir", where milk constitutes the initial fermenting substrate. This mixed culture consists of various yeasts (*Kluyveromyces*, *Candida*, *Saccharomyces*, and *Pichia*), various lactic acid bacteria of the genus *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and acetic acid bacteria (Garofalo et al., 2015; Kesmen & Kacmaz, 2011; Leite et al., 2012). Yeasts and lactic acid bacteria co-exist in a symbiotic association and are responsible for an acid-alcoholic fermentation. Kefir culture has been proposed for fermentation-upgrade of agro-industrial wastes (Kourkoutas et al., 2002; Plessas et al., 2008), as a starter in cheese production (Dimitrellou, Kandylis, Kourkoutas, Koutinas, & Kanellaki, 2015; Kourkoutas et al., 2006; Koutinas et al., 2010), and recently for fermentation of various vegetable and fruit juices (Corona et al., 2016; Randazzo et al., 2016). However, to the best of the authors' knowledge, it has not be tested for alcoholic and/or ML fermentations of cider.

Hence, the aim of the present study was to assess application of free or immobilized kefir culture on apple pieces and DCM in cider fermentations. Data documenting the ability of the kefir culture to conduct simultaneous alcoholic and ML fermentations successfully, avoiding biological competition among species, and quality improvement are presented.

2. Materials and methods

2.1. Kefir culture

Kefir starter culture was grown in synthetic medium consisting of 4% w/v glucose (Fluka, Switzerland), 0.4% w/v yeast extract (Fluka), 0.1% w/v $(NH_4)_2SO_4$ (Merck, Germany), 0.1% w/v KH_2PO_4 (Fluka), and 0.5% w/v $MgSO_4$ $7H_2O$ (Merck) at 30 °C for 24 h. The synthetic medium was sterilized at 121 °C for 15 min prior to use.

2.2. Apple juice

The concentrated apple juice used (100% apple juice Mr. Grand) was supplied by a local store. It had a pH value 3.1 and contained 112.8 g/L sugars, 0.1 g/L citric acid, 5.1 g/L malic acid, 0.3 g/L propionic acid, and 0.1 g/L glycerol.

2.3. Cell immobilization and determination of immobilized cell counts

Kefir culture was immobilized on apple pieces and DCM as previously described (Agouridis et al., 2008; Kourkoutas et al., 2001).

For the determination of the immobilized cell counts, 5 g of immobilized cells were blended with 45 mL of sterile ¼ Ringer's solution, serially diluted and subsequently plated. Then, lactobacilli, lactococci, and yeasts/molds counts were determined as described by Dimitrellou et al. (2008).

All analyses were performed in triplicate and the number of immobilized cells was determined as colony forming units (log cfu/g).

2.4. Fermentations

A series of repeated batch fermentations of commercial apple juice (250 mL) were carried out in batch bioreactors (0.5 and 1 L) using either free cells (10 g/L) or immobilized cells (480 g/L DCM and 1420 g/L apple pieces). Two separate experiments were carried out as follows: experiment A: a series of 13 repeated batch fermentations were performed at 30, 20, and 5 °C; experiment B: a series of 11 repeated batch fermentations were performed at 30, 37, and 45 °C.

All fermentations were carried out until all sugar content was utilized or when no fermentation activity was observed (stuck fermentations). Both free and immobilized cells were washed twice with apple juice and reused in the next batch fermentation. At the end of each batch fermentation, samples were collected and analyzed for residual sugars, ethanol, glycerol, organic acids, and volatile by-products.

2.5. Molecular analyses

2.5.1. PCR-DGGE analysis on biocatalysts

Samples of free (5 mL) or immobilized (5 g) cells were collected after fermentations at various temperatures and homogenized with 45 mL sterilized buffered peptone water (Lab M). Debris was allowed to deposit for 1 min and 1 mL of supernatant was used for DNA extraction using a DNeasy Tissue Kit (Qiagen, Germany), according to the manufacturer's protocol. PCR-DGGE analysis was performed, as previously described (Sidira, Galanis, Nikolaou, Kanellaki, & Kourkoutas, 2014; Sidira, Karapetsas, Galanis, Kanellaki, & Kourkoutas, 2014).

Bacterial DNA was amplified with primers V3f (5′ CCT ACG GGA GGC AGC AG 3′) and V3r (5′ ATT ACC GCG GCT GCT GG 3′) (Kesmen & Kacmaz, 2011; Pepe, Blaiotta, Moschetti, Greco, & Villani, 2003), while for eukaryotic DNA amplification primers NL1 (5′ GCC ATA TCA ATA AGC GGA GGA AAA G3′) and LS2 (5′ ATT CCC AAA CAA CTC GAC TC 3′) (Cocolin, Bisson, & Mills, 2000) were used. The PCR products were subjected to DGGE analysis using an INGENYphorU DGGE system (Ingeny, The Netherlands) (Sidira, Galanis et al., 2014; Sidira, Karapetsas et al., 2014). Followed the electrophoresis, the gels were scanned with a fluorescent imager (Molecular Imager FX, BioRad) and the bands of interest were excised.

2.5.2. Sequencing of DGGE fragments and data analysis

Sequencing of DGGE fragments and data analysis was carried out as previously described (Sidira, Galanis et al., 2014; Sidira et al., 2014).

2.6. Chemical analyses

2.6.1. pH, total and volatile acidity

pH was determined using a pH-330i pH meter (WTW GmbH, Germany). Total acidity was estimated by titration with 4 g/L NaOH solution and volatile acidity by titration with 4 g/L NaOH after steam distillation using an Electronic Distiller (DUALSTILL Exacta + Optech Labcenter S.p.a., Italy).

2.6.2. Determination of residual sugars, ethanol, glycerol, and organic acids concentration

Residual sugars (fructose & glucose), ethanol, glycerol, and organic acids (malic, lactic, acetic, citric, and propionic acids) concentration was determined by HPLC, using a Shimadzu chromatography system (Shimadzu Corp., Germany) equipped with a Nucleogel ION 300 OA column (Macherey-Nagel, Germany), a DGU-20A5R degassing unit, a LC-20AD pump, a CTO-20AC oven at 85 °C, and an RID-10A refractive index detector. A solution of 0.049 g/L

H_2SO_4 was used as mobile phase at 0.3 mL/min. The detector cell temperature was set at 60 °C. Twenty μL of each sample were injected directly to the column after double filtration with 0.22 μm filters. Residual sugars, ethanol, glycerol, and organic acids concentrations were calculated using standard curves prepared by standard solutions ($R^2 \geq 0.99$).

Ethanol productivity was expressed as g of ethanol produced per day per liter of liquid volume of bioreactor and conversion was calculated by the following equation: (Initial sugar conc.-Residual sugar conc.)/Initial sugar conc. × 100. Malic acid conversion was calculated by the equation: (Initial malic acid conc.-Residual malic acid conc.)/Initial malic acid conc. × 100. Ethanol production yield was expressed as g of ethanol produced per g of sugars utilized.

2.6.3. Volatile by-products determination

2.6.3.1. Major volatile by-products determination. Major volatile by-products (acetaldehyde, ethyl acetate, 1-propanol, isobutanol, 1-hexanol, amyl alcohol, isoamyl alcohol, and methanol) were analyzed using a MASTER GC Fast Gas Chromatograph (DANI Instruments S.p.a., Italy) equipped with a MASTER AS Liquid Autosampler (DANI Instruments S.p.a.), an FID detector, and a CP-Wax 57 CB column (50 m × 0.32 mm i.d., 0.20 μm film thickness, Chrompack). Sample analysis was carried out as previously described (Moreira et al., 2011) with some modifications. Briefly, samples (1 μL) were directly injected into the column. Oven temperature was set at 40 °C, held constant for 5 min, followed by a raise to 180 °C at a 3 °C/min rate. The injector was operated in split mode (1:20). Both injector and detector temperatures were set at 250 °C. Results were processed by Clarity MASTER GC Communication integrated software (DANI Instruments S.p.a.). Concentration of volatile compounds, was calculated using standard curves prepared by standard solutions ($R^2 \geq 0.99$).

2.6.3.2. HS-SPME GC/MS analysis. All batch fermentations carried out at 20 and 37 °C were subjected to HS-SPME GC/MS analysis. The HS-SPME GC/MS analysis was carried out as previously described (Kandylis, Drouza, Bekatorou, & Koutinas, 2010) using a GC/MS (6890N GC, 5973NetworkedMS MSD, Agilent Technologies, USA) equipped with an HP-5MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness). Results were processed by ChemStation integrated software (Agilent Technologies). The identification was carried out by comparing the retention times and mass spectra of volatiles to those of authentic compounds (ethyl acetate, ethyl 2-methyl-butyrate, ethyl 3-methyl-butyrate, 3-methylbutyl acetate, 2-methylbutyl acetate, ethyl hexanoate, hexyl acetate, ethyl octanoate, 2-phenylethyl acetate, ethyl nonanoate, ethyl 9-decenoate, ethyl decanoate, ethyl dodecanoate, ethyl hexadecanoate, octanoic acid, decanoic acid, 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-hexanol, benzyl alcohol, 3,7-dimethyl-1,6-octadien-3-ol, 2-phenylethanol, 3,7-dimethyl-6-octen-1-ol, 3,7,11-trimethyl-1,6,10-dodecatrien-3-ol, hexanal, benzaldehyde, 1,1-diethoxy-ethane, toluene, styrene, pentadecane) generated in the laboratory (*in-house* libraries), by mass spectra obtained from NBS75K and Wiley275 libraries, and by determining kovats' retention indexes and comparing them with those reported in the literature (data non shown). 4-methyl-2-pentanol diluted in pure ethanol was used as an internal standard (IS). The volatile compounds were semi-quantified by dividing the peak areas of the compounds of interest by the peak area of the IS and multiplying this ratio by the initial concentration of the IS (expressed as mg/L). The peak areas were measured from the full scan chromatograph using total ion current (TIC).

2.7. Preliminary sensory evaluation

Cider products were evaluated for their sensory characteristics (aroma, taste, and overall quality) and compared with two similar type commercial products [Strongbow cider gold apple 4.5% vol. (H. P. Bulmer Ltd., London, UK) and Somersby 4.5% vol. (Carlsberg Group, Copenhagen, Denmark)]. Twelve random tasters familiar with cider products were asked to give scores on a 0–5 scale using locally approved protocols in our laboratory, as previously reported (Tsakiris et al., 2006). The sensory evaluation was a blind test in a coloured glass under low light and all samples were served at 12–15 °C.

2.8. Statistical analysis

Data were analyzed for statistical significance with analysis of variance (ANOVA). Specifically, two-way ANOVA was applied for fermentation parameters and concentrations of organic acids and volatiles, while one-way ANOVA in the preliminary sensory evaluation. Duncan's multiple range test was used to determine significant differences among mean values (coefficients, ANOVA tables, and significance at $P < 0.05$ were computed using Statistica v.10.0).

Principal component analysis (PCA) was computed using XLSTAT 2015.1. The cider data set consisted of a 7 × 64 matrix, in which rows (7 rows) represented the cider samples and columns (64 columns) the instrumental analysis values. Each sample was represented in the multi-dimensional space by a data vector, which was an assembly of the 64 features in cider samples. Data vectors belonging to the same category (nature of kefir culture, ap, DCM, fr, ap_j; fermentation temperature, 20, 37 °C) were analyzed using chemometric procedures.

3. Results & discussion

3.1. Cell immobilization and fermentations

The rational of the present study was to assess free and immobililzed kefir culture for conducting simultaneous alcoholic and ML fermentations, avoiding species competition and leading in significant technological and quality improvements in cider-making.

Initially, kefir culture was successfully immobilized on apple pieces and DCM. Microbiological analysis showed that viable counts of immobilized kefir culture on apple pieces consisted of 7.74 ± 0.10 logcfu/g yeasts/molds, 6.0 ± 0.10 logcfu/g lactobacilli, and 6.0 ± 0.10 logcfu/g lactococci, whereas the corresponding values for immobilized cells on DCM were 7.65 ± 0.05 logcfu/g yeasts/molds, 6.15 ± 0.15 logcfu/g lactobacilli, and 6.3 ± 0.10 logcfu/g lactococci.

To monitor the fermentation efficiency of both free and immobilized kefir culture in a wide temperature range, fermentations were performed at ambient and low temperatures (5–30 °C, experiment A), as well as at ambient and high temperatures (30–45 °C, experiment B). The results are presented in Tables 1 and 2.

Both the fermentation temperature and the nature of kefir culture (free or immobilized) affected significantly ($P < 0.05$) the fermentation time, ethanol productivity, and concentration of citric acid and a strong interaction ($P < 0.05$) between the two factors was obvious. Similarly, the amount of glycerol, ethanol production yield, and pH were affected ($P < 0.05$) by both factors, but no interaction was recorded ($P > 0.05$). On the other hand, total acidity was only affected ($P < 0.05$) by the fermentation temperature, while no significant differences were observed in malic acid content and conversion ($P > 0.05$), although a significant ($P < 0.05$) interaction between the two factors was observed. Likewise, ethanol content,

Table 1
Fermentation parameters of ciders produced by repeated batch fermentations at a wide temperature range using free or immobilized kefir culture.

Nature of kefir culture/Factors	Fermentation Temperature (°C)	Repeated batch fermentations	Fermentation time (h)	Ethanol concentration (% vol.)	Glycerol (g/L)	Residual sugars (g/L)	Ethanol productivity [g/(Ld)]	Ethanol production yield	Conversion (%)	Total acidity (g malic/L)	Volatile acidity (g acetic/L)	pH
Experiment A												
Free cells	30	1–4	68–117	5.7–6.6	2.5–2.8	1.2–2.5	9.2–18.5	0.40–0.48	97.8–98.9	3.8–4.5	0.09–0.12	3.4
	20	5–9	87–140	6.4–6.6	2.5–3.3	1.9–3.0	8.6–14.5	0.46–0.48	97.3–98.3	4.6–5.3	0.09–0.18	3.4–3.5
	5	10–13	354–527	6.5–6.6	2.1–2.6	4.2–6.9	2.4–3.6	0.48–0.49	93.9–96.3	4.5–4.6	0.15–0.18	3.3–3.7
Immobilized cells on apple pieces	30	1–4	64–70	4.4–6.5	2.0–3.0	1.0–1.5	12.9–17.6	0.31–0.46	98.6–99.1	4.2–4.7	0.09–0.12	3.4–3.5
	20	4–9	96–192	6.1–6.6	2.6–3.0	0.6–3.9	6.1–13.0	0.43–0.48	96.5–99.5	4.8–5.4	0.09–0.12	3.3–3.4
	5	10–13	932–1216	3.8–6.6	1.5–2.9	1.1–7.1	0.6–1.3	0.28–0.49	93.7–99.0	2.4–3.8	0.15–0.18	3.3–3.5
Immobilized cells on DCM	30	1–4	43–70	5.1–6.3	2.9–3.1	1.1–2.1	15.5–28.0	0.36–0.45	98.1–99.0	4.3–4.8	0.09–0.18	3.4–3.5
	20	5–9	90–119	6.4–6.6	3.0–3.4	1.0–1.6	10.5–14.0	0.46–0.47	98.6–99.2	4.5–5.3	0.09	3.4–3.5
	5	10–13	469–600	5.7–6.6	2.4–3.9	2.4–9.8	2.1–2.6	0.41–0.49	91.3–97.8	3.1–4.2	0.12–0.18	3.4–3.5
Experiment B												
Free cells	30	1–4	51–142	6.3–6.6	2.6–2.8	1.1–2.4	8.8–23.5	0.45–0.47	97.8–99.0	4.5–5.1	0.09–0.12	3.3–3.4
	37	5–9	41–52	6.5–6.6	2.8–3.0	1.7–2.7	23.8–30.3	0.46–0.47	97.6–98.5	3.9–4.7	0.09–0.15	3.4–3.5
	45	10–11	87–92	1.7–5.1	1.3–2.7	27.7–84.2	3.5–11.1	0.47	25.4–75.5	3.5–3.8	0.09–0.12	3.5–3.7
Immobilized cells on apple pieces	30	1–4	44–70	5.7–6.4	2.8–3.4	1.3–2.1	17.3–27.2	0.40–0.45	98.2–98.9	4.0–5.0	0.09–0.12	3.3–3.4
	37	5–9	44–71	5.9–6.6	2.5–3.1	2.3–4.1	17.3–26.1	0.42–0.48	96.3–98.0	4.0–4.8	0.09–0.12	3.3–3.4
	45	10–11	140–141	1.5–5.0	0.8–2.6	30.2–73.0	2.0–6.8	0.30–0.48	35.3–73.2	3.5–3.8	0.09–0.15	3.4–3.5
Immobilized cells on DCM	30	1–4	43–67	5.4–6.6	2.6–3.7	0.9–1.5	15.3–29.3	0.38–0.47	98.7–99.2	4.2–5.3	0.09–0.39	3.5
	37	5–9	22–24	6.1–6.6	3.0–3.5	2.3–3.2	52.2–56.9	0.44–0.48	97.2–98.0	4.2–4.8	0.09	3.4–3.5
	45	10–11	90–113	2.3–5.9	1.2–3.2	8.7–53.8	4.8–9.8	0.30–0.45	52.3–92.3	3.2–4.0	0.09	3.5–3.6
***F*-values**												
Nature of kefir culture	–	–	73.67**	1.70	7.94**	2.42	29.39**	5.98**	2.42	1.12	0.52	5.10**
Fermentation temperature	–	–	545.58**	19.06**	7.27**	42.09**	160.60**	3.70**	42.06**	26.10**	4.87**	4.8**
Interaction	–	–	55.73**	0.38	0.82	1.24	18.55**	0.80	1.23	3.64**	1.70	1.40

Table 2
Organic acids profile of ciders produced by repeated batch fermentations at a wide temperature range using free or immobilized kefir culture.

Nature of kefir culture/Factors	Fermentation Temperature (°C)	Repeated batch fermentations	Malic acid (g/L)	Lactic acid (g/L)	Malic acid conversion (%)	Acetic acid (g/L)	Citric acid (g/L)	Propionic acid (g/L)
Experiment A								
Free cells	30	1–4	4.3–4.9	0.2–0.4	3.5–15.1	0.1–0.2	4.7–6.1	0.2–0.3
	20	5–9	2.4–4.6	0.1–0.3	9.0–52.4	0.2–0.3	4.1–5.3	0.3–0.4
	5	10–13	4.2–4.7	0.1–0.3	7.6–18.1	0.4–0.7	4.7–5.8	0.3–0.4
Immobilized cells on apple pieces	30	1–4	1.5–3.7	0.2–0.6	27.6–71.5	0.1–0.2	2.8–4.4	0.2–0.3
	20	5–9	3.7–5.0	0.2–0.5	2.0–27.4	0.2–0.3	2.8–3.9	0.3–0.4
	5	10–13	2.4–5.1	0.2–0.4	<0.1–52.6	0.3–0.7	1.8–5.4	0.2–0.5
Immobilized cells on DCM	30	1–4	3.0–4.7	0.4–1.3	8.4–41.3	0.1–0.5	3.8–5.1	0.3
	20	5–9	3.8–4.8	0.2–0.4	6.3–26.2	<0.1–0.2	5.0–5.4	0.3–0.4
	5	10–13	3.4–5.1	0.1–0.2	0.1–33.6	0.5–0.7	4.2–7.3	0.1–0.5
Experiment B								
Free cells	30	1–4	4.6–4.9	0.3–0.5	3.8–9.6	0.2	5.2–6.2	0.3–0.4
	37	5–9	2.0–4.8	0.2–0.4	6.2–60.9	0.2–0.3	5.4–6.1	0.3–0.4
	45	10–11	3.9–4.9	0.1–0.2	4.4–23.9	0.2–0.3	5.7–6.6	0.4
Immobilized cells on apple pieces	30	1–4	2.3–4.2	0.2–0.6	17.6–55.0	0.1–0.3	4.2–4.5	0.3
	37	5–9	3.7–4.2	0.2–0.3	19.0–28.4	0.1–0.2	4.2–5.0	0.3
	45	10–11	3.9–4.1	0.0–0.1	18.7–23.9	0.1–0.3	5.1–5.8	0.3–0.4
Immobilized cells on DCM	30	1–4	2.1–4.2	0.4–2.5	17.0–59.8	0.1–1.3	3.6–4.7	0.3
	37	5–9	4.2–4.6	0.3–0.4	10.3–17.4	0.1–0.2	5.2–5.6	0.3–0.4
	45	10–11	3.4–3.9	0.1–0.2	24.0–33.6	0.1–0.2	5.7–7.0	0.3
***F*-values**								
Nature of kefir culture	–	–	2.01	2.19	1.94	0.75	22.34**	2.76
Fermentation temperature	–	–	1.30	7.15**	1.39	0.93	6.74**	3.03*
Interaction	–	–	3.01**	1.96	2.99**	0.93	2.92**	0.58

*$P < 0.05$, **$P < 0.01$.

residual sugars, conversion, volatile acidity, as well as lactic and propionic acids were only affected ($P < 0.05$) by the fermentation temperature. In contrast, no significant differences were noted in acetic acid concentration ($P > 0.05$).

Fermentations were continued for 13 and 11 repeated batches in experiment A and B, respectively, indicating a high operational stability of the systems (for a period higher than 7 months). Low fermentation temperatures (5 °C) resulted in low ($P < 0.05$) fermentation rates, whereas high temperatures (45 °C) led to significantly ($P < 0.05$) higher residual sugars and significantly ($P < 0.05$) reduced ethanol content and conversion values. High ethanol productivities were recorded at 30 and 37 °C. Noticeably, the highest ($P < 0.05$) values [up to 56.9 g/(Ld)] were observed in fermentations with immobilized cells on DCM at 37 °C, which were considered similar or even higher than in industrial practice, as fermentations were completed in less than 24 h (Kopsahelis, Bosnea, Kanellaki, & Koutinas, 2012).

Malic acid conversion ranged up to 71.5% and was similar to values previously published (Durieux, Nicolay, & Simon, 2000; Nedovic et al., 2000). The degradation of malic acid and the consequent lowering of total acidity is a major demand in cider production. Our systems provided a medium degradation rate, but long operational stability. High content of lactic acid does not contribute positively to quality, as it results in increased volatile acidity. The highest ($P < 0.05$) amounts of lactic acid were determined in cider fermented by immobilized kefir culture on DCM at 30 °C and especially at the first batch (2.5 g/L) and it was significantly reduced in subsequent fermentations. Similar content of lactic acid (1.87 g/L) after ML fermentation of wine has been previously reported by Agouridis et al. (2008). However, volatile acidity ranged in very low levels in all cases.

3.2. Determination of kefir culture biodiversity using PCR-DGGE analysis

Samples of free or immobilized cells were collected after fermentations at various temperatures and subjected to PCR-DGGE analysis to monitor potential changes in microbial diversity during cell immobilization and fermentations. The results are summarized in Table 3 and Fig. 1. Assays were conducted in triplicate and identical profiles were obtained for the same samples. Sequence determination of the separated bands revealed similar microbial patterns in all cases. Members of the *Lactobacillus* species (*L. helveticus, L. dextrinicus*, *L. buchneri*, *L. kefiri*, and *L. sunkii*) consisted the predominant bacteria populations, whereas *Klyveromyces marxianus*, *Klyveromyces lactis*, and *Saccharomyces cerevisiae* were the main yeasts identified.

Taking into account that the detection limit for PCR-DGGE identification in mixed cultures is 3–4 logcfu/g (Cocolin, Manzano, Cantoni, & Comi, 2001) when the predominant populations are above 8 logcfu/g and may reach up to 7–8 logcfu/g (Ercolini, 2004), the existence of other microbial species can not be excluded. The detection limit also depends on the species and perhaps even the strain considered. Moreover, the number and the concentration of the other members of the microbial community, along with the nature of the food matrix, all represent variables influencing the detection limit of DGGE by affecting both the efficiency of DNA extraction and the PCR amplification, due to the possible competition among templates (Ercolini, 2004). In addition,

Table 3
Phylogenetic affiliations of microbial dynamics in free or immobilized kefir culture after cider fermentations at various temperatures based on DNA analyses and the corresponding bands in the DGGE profile.

Band	Most closely related species	Identity (%)	Accession Number
B1	*Uncultured Lactobacillus* sp.	97	LT007073.1
	Uncultured bacterium	97	LT008864.1
	Lactobacillus helveticus	97	KP763889.1
	Lactobacillus dextrinicus	97	LN870301.1
B2/B3	*Lactobacillus kefiri*	97	KC964542.1
	Lactobacillus buchneri	97	KC336485.1
	Lactobacillus sunkii	97	JF965394.1
Y1	*Kluyveromyces marxianus*	96	FJ896141.1
	Kluyveromyces lactis	98	KJ183045.1
Y2	*Saccharomyces cerevisiae*	97	HM107794.1

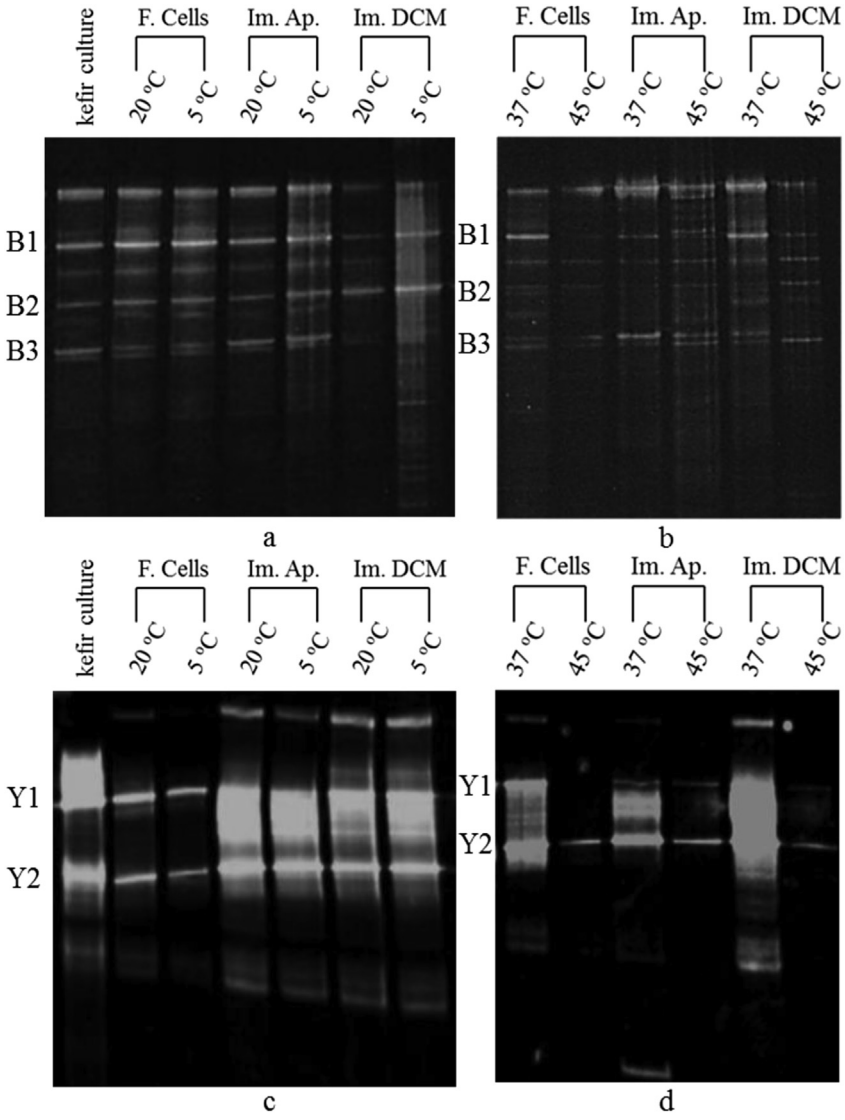

Fig. 1. DGGE bacterial (a, b) and eukaryotic (c, d) fingerprint representing PCR-amplified 16S rRNA and 26S rRNA, respectively, from total community DNA derived from free or immobilized kefir culture before and after cider fermentation at a wide temperature range. For each sample, two replicate profiles from two independent nucleic acid extracts were analyzed. All bands marked by letters were subjected to sequence determination. F. Cells: Free kefir culture, Im. Ap: Kefir culture immobilized on apple pieces, Im. DCM: Kefir culture immobilized on DCM.

the V3 region of the 16S rRNA gene seems to contain insufficient differences for the separation in DGGE of closely related species, such as *L. buchneri*, *L. kefiri*, and *L. sunkii* (Garofalo et al., 2015; Kesmen & Kacmaz, 2011). Other potential reasons might have been masking effects by DNA belonging to the major populations present or poor amplification of the above species by the protocol applied (Rantsiou et al., 2005).

Our results were in accordance to other studies (Garofalo et al., 2015; Kesmen & Kacmaz, 2011; Leite et al., 2012; Sabir, Beyatli, Cokmus, & Onal-Darilmaz, 2010). However, *L. kefiranofaciens*, a species usually present in kefir grains (Garofalo et al., 2015; Leite et al., 2012) was not identified in our culture. Although bacteria and yeasts in kefir grains originating from different regions should not vary significantly, kefir grains may change their microbial make up and fermentation properties over time and under different growing conditions (Leite et al., 2012).

3.3. Volatiles

The development of a unique aromatic profile is an undeniable aim for the cider industry. Thus, analysis of both major and minor volatile byproducts, which define cider flavor, was performed.

3.3.1. Major volatiles

The results of the major volatiles are presented in Table 4. Both the fermentation temperature and the nature of kefir culture (free or immobilized) affected significantly ($P < 0.05$) the concentration of acetaldehyde, ethyl acetate, 1-propanol, isobutanol, 1-hexanol, amyl alcohol, isoamyl alcohol, and methanol, while a strong interaction between the two factors was observed in acetaldehyde, ethyl acetate, and methanol content.

The acetaldehyde content in ciders usually ranges up to 120 mg/L (legal limit in France). Higher amounts (>150 mg/L) are associated with spoilage, called “framboisé” (cider-sickness) (Lachenmeier & Sohnius, 2008). In the present study, acetaldehyde concentrations up to 106 mg/L were observed. However, in most cases they were lower (Table 4).

Generally, cell immobilization and low fermentation temperatures (5 °C) resulted in increased ethyl acetate content, affecting positively cider quality. Noticeably, the highest ($P < 0.05$) ethyl

Table 4
Major volatiles of cider produced by repeated batch fermentations at a wide temperature range using free or immobilized kefir culture.

Nature of kefir culture/Factors	Temperature (°C)	Repeated batch fermentations	Acetaldehyde (mg/L)	Ethyl acetate (mg/L)	1-Propanol (mg/L)	Isobutanol (mg/L)	1-Hexanol (mg/L)	Amyl alcohol (mg/L)	Isoamyl alcohol (mg/L)	Methanol (mg/L)
Experiment A										
Free cells	30	1–4	10–22	0–8	6–9	24–33	0–1	13–15	47–51	0
	20	5–9	4–28	4–12	6–10	18–28	0–2	9–16	39–70	0
	5	10–13	44–56	7–18	5–8	8–12	0–1	5–7	19–32	0–5
Immobilized cells on apple pieces	30	1–4	3–9	8–40	7–13	32–51	1–2	11–17	43–58	0–12
	20	5–9	2–11	13–20	8–9	28–39	1–2	11–15	54–76	0–4
	5	10–13	5–21	13–59	4–14	6–26	1–3	3–12	14–61	0–13
Immobilized cells on DCM	30	1–4	6–25	12–33	13–18	26–45	1–2	12–27	45–97	4–22
	20	5–9	2–40	15–32	9–22	29–66	1–3	15–36	67–150	0–6
	5	10–13	13–44	68–90	14–24	23–49	2–5	15–29	63–129	3–5
Experiment B										
Free cells	30	1–4	19–34	13–15	13–14	42–61	2–4	27–30	95–101	0–3
	37	5–9	5–31	6–13	8–22	37–90	1–3	15–30	51–94	0–4
	45	10–11	18–106	4	4–5	20–32	2–4	10	16–24	0–3
Immobilized cells on apple pieces	30	1–4	5–11	20–53	17–21	77–95	3–4	29–34	98–126	5–10
	37	5–9	8–36	7–20	12–34	48–119	1–4	15–38	44–112	0–6
	45	10–11	30–32	4–5	8–9	14–24	2–3	2–8	14–23	8
Immobilized cells on DCM	30	1–4	8–55	21–30	16–28	20–74	3–9	8–40	27–138	13–40
	37	5–9	13–25	9–19	17–30	48–133	2–5	21–42	64–117	4–10
	45	10–11	47–71	5–10	8–22	35–86	2–4	12–28	29–68	5–6
***F*-values**										
Nature of kefir culture	–	–	10.21**	29.65**	17.03**	6.00*	4.47*	9.34**	9.03**	7.45**
Fermentation temperature	–	–	10.90**	25.12**	11.06**	20.62**	2.64*	11.69**	8.21**	5.79**
Interaction	–	–	2.17*	10.79**	0.78	1.48	0.50	0.98	1.14	3.96**

*$P < 0.05$, **$P < 0.01$.

Table 5
Minor volatile compounds (mg/L) identified in ciders produced by free or immobilized kefir culture at 20 and 37 °C using the HS-SPME GC/MS analysis. The concentration of volatiles were semi-quantified using 4-methyl-2-pentanol as internal standard.

Compounds	KI	20 °C			37 °C			Apple juice	Factors	*F*-values	Potential origin
		Free kefir culture	Kefir immob. on apple pieces	Kefir immob. on DCM	Free kefir culture	Kefir immob. on apple pieces	Kefir immob. on DCM				
Esters											
Ethyl acetate	<700	1.1–3.0	1.9–4.5	2.4–4.3	1.2–1.7	1.3–2.5	1.2–2.0	0.1			present in apples
Ethyl propanoate	707	0.0–0.1	0.0–0.3	0.0–0.1	0.0–0.1	0.0–0.2	0.0–0.1	0.0-<0.1			yeast fermentation by-product; present in apples
Isobutyl acetate	745	Nd	0.0–0.1	0.0-<0.1	Nd	0.0–0.1	0.0–0.1	Nd			present in apples
Ethyl butyrate	803	0.4–0.8	0.0–1.1	0.6–0.8	0.5–0.7	0.6–1.2	0.1–0.9	0.1–2.3			yeast fermentation by-product; present in apples
Butyl acetate	812	Nd	Nd	Nd	Nd	Nd	Nd	0.0–0.1			unknown
Ethyl 2-methyl-butyrate	841	0.4–0.6	0.0–1.0	0.4–0.6	0.3–0.4	0.2–0.7	0.0–0.3	Nd			yeast fermentation by-product
Ethyl 3-methyl-butyrate	845	0.0–0.1	0.0–0.2	0.0–0. 1	0.0–0.1	0.0–0.1	0.0–0. 1	0.0–0.2			yeast fermentation by-product
3-methylbutyl acetate (Isoamyl acetate)	867	3.3–6.2	3.4–6.1	4.5–5.9	2.9–3.6	1.8–4.4	2.6–4.1	<0.1–6.1			present in cider
2-methylbutyl acetate	869	0.0–0.6	0.0–0.6	0.5–0.6	0.4–0.5	0.0–0.6	0.4–0.6	0.2–1.2			unknown
Ethyl hexanoate	1002	7.0–11.0	15.5–36.3	7.1–13.0	3.4–5.7	4.8–19.1	4.4–12.2	0.0-<0.1			yeast fermentation by-product; present in apples
(Z)-3-hexen-1-ol acetate	1009	Nd	0.0–2.8	0.0–6.0	0.0–8.1	0.0–3.3	0.0–6.1	Nd			unknown
Hexyl acetate	1018	3.4–6.9	0.9–2.8	3.9–6.6	4.3–6.7	1.0–2.5	3.1–7.2	0.1–16.5			yeast fermentation by-product; present in apples
Ethyl 2-hexenoate	1053	0.0–0.1	0.0–0.1	0.0–0.1	0.0–0.1	0.0–0.1	0.0–0.1	Nd			yeast fermentation by-product
Ethyl heptanoate	1126	Nd	0.0–1.1	0.0-<0.1	Nd	0.0–0.3	Nd	Nd			yeast fermentation by-product
Isobutyl hexanoate	1169	Nd	0.0–0.3	Nd	Nd	0.0–0.1	0.0–0.1	Nd			unknown
Phenylmethyl acetate	1175	0.0–0.6	0.0–0.3	0.0–0.6	0.5–1.4	0.2–0.4	0.1–1.2	0.0–2.9			present in apples
Diethyl butanedioate	1191	0.0–0.5	0.0–1.8	0.0–0.5	0.0–0.6	0.0–0.9	0.0–0.4	Nd			fermentation by-product
Ethyl octanoate	1202	42.2–81.5	52.4–206.2	62.8–117.6	25.0–50.1	32.9–160.0	29.4–95.2	Nd			yeast fermentation by-product; present in apples
Hexyl 2-methyl-butyrate	1244	Nd	0.0–0.3	0.0-<0.1	Nd	0.0–0.5	0.0-<0.1	Nd			unknown
2-methylbutyl hexanoate	1259	0.0–0.1	0.0–0.6	0.0–0.2	0.0–0.1	Nd	0.0–0.1	Nd			unknown
2-phenylethyl acetate	1263	0.4–0.9	1.2–10.2	0.6–1.3	0.3–0.8	0.3–1.0	0.5–1.7	Nd			yeast fermentation by-product; present in apples
Benzyl isobutyrate	1305	0.0–0.1	0.0–0.1	0.0–0.1	0.0–0.2	0.0–0.3	0.0–0.2	0.0–0.9			unknown
Ethyl nonanoate	1307	0.0–0.1	0.0–0.4	0.0–0.1	0.0–0.1	0.0–0.3	0.0–0.2	Nd			yeast fermentation by-product
Isobutyl octanoate	1354	Nd	0.0–0.5	Nd	Nd	0.0–0.3	0.0–0.2	Nd			unknown
(*Z*)-3,7-dimethyl-2,6-octadien-1-ol acetate (neryl acetate)	1386	0.0–0.2	0.0–0.1	0.0–0.3	0.2–0.6	Nd	0.3–0.7	0.0–1.4			unknown
Ethyl 9-decenoate	1390	2.9–7.8	2.5–17.9	2.7–11.5	0.8–1.5	1.4–4.5	0.9–3.3	Nd			yeast fermentation by-product
Ethyl decanoate	1398	5.2–12.9	7.3–60.1	7.7–30.5	9.5–21.7	11.2–36.0	12.0–26.2	0.0–0.1			yeast fermentation by-product
3-methylbutyl octanoate	1453	0.0–0.4	0.1–1.5	0.0–0.8	0.0–0.2	0.0–0.7	0.0–0.3	Nd			yeast fermentation by-product
Ethyl dodecanoate	1595	0.1–0.7	0.2–6.0	0.5–2.8	0.5–1.2	1.5–3.4	0.7–1.7	Nd			yeast fermentation by-product
3-methylbutyl decanoate	1646	Nd	0.0–0.2	0.0–0.1	0.0–0.1	0.0–0.1	0.0–0.1	Nd			unknown
Ethyl hexadecanoate	1995	0.0–0.6	0.2–0.9	0.1–1.1	0.0–0.3	0.0–0.4	<0.1–0.2	Nd			yeast fermentation by-product
Ethyl linoleate	2175	0.0–0.3	0.1–0.5	<0.1–0.4	0.0–0.2	0.0–0.2	0.0–0.2	Nd			unknown
Total esters		**71.5–131.2**	**89.6–325.8**	**102.9–197.5**	**55.8–105.0**	**61.6–243.4**	**63.2–153.6**	**0.7–29.5**	Nature of kefir culture	7.19**	–
									Fermentation temperature	4.80*	–
									Interaction	1.61	–
Organic acids											
Octanoic acid	1198	0.0–2.2	0.0–3.4	0.9–2.6	0.9–1.8	0.1–3.0	0.0–1.9	Nd			present in apples
Decanoic acid	1381	0.0–0.2	0.9–1.7	0.3–0.7	0.4–1.8	0.6–1.5	0.2–2.0	Nd			present in apples

Total organic acids		**0.0–2.2**	**1.7–4.6**	**1.2–3.2**	**1.3–2.9**	**1.3–5.0**	**0.8–3.3**	**Nd**	Nature of kefir culture	3.02	–
									Fermentation temperature	0.26	–
									Interaction	1.00	–
Alcohols											
1-propanol	<700	Nd	0.0–0.1	0.0–0.1	0.0–0.1	0.0–0.2	0.0–0.3	Nd			present in apples; yeast fermentation by-product
2-methyl-1-propanol (isobutanol)	<700	0.2–0.5	0.0–1.1	0.5–1.3	0.5–0.9	0.6–1.4	0.6–1.6	0.0-<0.1			present in apples; yeast fermentation by-product
3-methyl-1-butanol (isoamyl alcohol)	721	10.9–21.1	13.2–31.6	19.1–34.2	12.1–21.9	13.6–25.6	13.1–21.1	0.1–0.2			present in apple juice; yeast fermentation by-product
2-methyl-1-butanol (amyl alcohol)	722	1.7–6.0	2.3–7.7	3.5–10.8	3.9–7.5	3.5–8.4	4.2–8.4	0.0–0.3			present in apple juice; yeast fermentation by-product
(*Z*)-3-Hexen-1-ol	846	0.4–1.3	0.0–1.6	0.6–1.2	0.6–1.7	0.5–1.6	0.0–1.1	0.0–1.1			present in cider; fermentation by-product
(*E*)-2-Hexen-1-ol	857	Nd	Nd	Nd	Nd	Nd	Nd	0.0–0.2			unknown
1-Hexanol	861	0.9–1.9	0.0–3.4	1.5–2.0	1.3–2.6	1.4–3.5	1.1–2.0	0.9–2.6			fermentation by-product; present in apples
Benzyl alcohol	1036	0.0–0.2	0.0–0.6	0.0–0.4	0.0–0.2	0.0–0.8	0.0–0.4	0.0–0.3			present in cider
3,7-dimethyl- 1,6-octadien-3-ol (linalool)	1123	0.0–0.2	0.0–0.4	0.0–0.2	0.0–0.3	0.0–0.5	0.0–0.2	0.0–0.5			unknown
2-phenylethanol	1133	0.6–4.7	3.8–8.4	3.7–5.8	5.0–8.5	3.2–10.3	1.8–6.5	Nd			yeast fermentation by-product
2-ethyl-1-pentanol	1156	Nd	0.0–0.1	Nd	Nd	Nd	Nd	0.0-<0.1			unknown
3,7-dimethyl-6-octen-1-ol (citronellol)	1235	0.0–0.6	0.0–0.7	0.0–0.3	0.3–0.7	0.0–0.6	0.0–0.3	0.0–0.1			fermentation by-product
1-undecanol	1282	0.0–0.1	0.0–0.2	Nd	Nd	0.0–0.3	0.0–0.1	Nd			unknown
3,7,11-trimethyl-1,6,10-dodecatrien-3-ol (nerolidol)	1565	0.0–0.1	0.0–0.1	0.0–0.1	0.0–0.3	0.0–0.4	0.0–0.1	Nd			unknown
Total alcohols		**18.3–35.0**	**22.9–55.0**	**32.3–53.2**	**24.9–40.4**	**26.5–53.4**	**25.2–36.3**	**2.1–3.4**	Nature of kefir culture	2.05	
									Fermentation temperature	0.03	
									Interaction	1.66	
Carbonyl compounds											
3-methyl-butanal	<700	Nd	Nd	Nd	0.0-<0.1	Nd	0.0-<0.1	Nd			present in apples
2-methyl-butanal	<700	Nd	Nd	Nd	Nd	Nd	0.0-<0.1	Nd			unknown
Hexanal	800	Nd	0.0-<0.1	Nd	Nd	Nd	0.0-<0.1	<0.1–0.3			unknown
Benzaldehyde	947	Nd	Nd	Nd	Nd	Nd	0.0–0.1	Nd			fermentation by-product
(*E*)-1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one (β-damascenone)	1384	0.0–0.1	0.0–0.4	0.0–0.1	0.0–0.1	0.0–0.4	0.0–0.2	<0.1–0.1			fermentation by-product
5-hexyldihydro-2(3H)-furanone (γ-decalactone)	1470	0.2–0.5	0.4–3.6	0.3–0.9	0.3–0.9	0.3–0.7	0.3–0.5	0.0–0.3			unknown
Total carbonyl compounds		**0.2–0.5**	**0.6–3.6**	**0.3–0.9**	**0.3–1.0**	**0.5–1.1**	**0.3–0.8**	**0.0–0.7**	Nature of kefir culture	2.65	–
									Fermentation temperature	0.43	–
									Interaction	2.42	–
Miscellaneous compounds											
2-fluoro-1-propene	<700	0.0–0.1	0.0–0.1	0.0–0.4	0.0–0.1	0.0–0.3	0.0–0.1	0.0-<0.1			unknown
2,5-dimethyl-furan	704	Nd	0.0-<0.1	0.0–0.1	0.0–0.1	0.0-<0.1	0.0-<0.1	Nd			unknown
1,1-diethoxy-ethane (acetal)	716	0.0–0.4	0.0–0.2	0.0–0.3	0.0–0.3	0.0–0.1	0.1–0.2	Nd			fermentation by-product
Toluene	735	0.0–0.1	0.0–0.1	Nd	0.0–0.1	0.0–0.1	0.0–0.1	<0.1–0.1			unknown
Styrene	873	Nd	0.0–0.1	0.0–0.1	Nd	0.0–0.1	0.0–0.1	Nd			unknown
1,3,5-trimethyl-benzene (mesitylene)	956	0.0–0.4	0.0–0.4	0.0–0.3	0.0–0.3	0.0–0.5	0.0–0.3	<0.1–0.2			unknown

(*continued on next page*)

Table 5 (*continued*)

Compounds	KI	20 °C			37 °C			Apple juice	Factors	*F*-values	Potential origin
		Free kefir culture	Kefir immob. on apple pieces	Kefir immob. on DCM	Free kefir culture	Kefir immob. on apple pieces	Kefir immob. on DCM				
2-methyl-benzaldehyde	1091	0.0–0.2	0.0–<0.1	0.0–0.4	0.0–0.5	Nd	0.0–<0.1	Nd			unknown
(1.alpha.,3.alpha.,6.alpha.)-3,7,7-trimethyl-bicyclo [4.1.0]heptane	1302	5.6–8.6	4.7–15.8	5.2–11.1	5.0–8.8	3.7–11.2	0.9–7.2	0.0–9.2			unknown
Pentadecane	1503	0.0–0.1	0.0–0.1	0.0–0.1	0.0–0.1	0.0–0.1	0.0–0.1	Nd			unknown
2,4-bis(1,1-dimethylethyl)-phenol	1516	0.0–1.1	0.0–0.9	0.0–0.5	0.0–0.5	0.0–0.5	0.0–0.3	0.0–0.2	–	–	unknown
Total miscellaneous compounds		**6.1–9.7**	**6.1–15.8**	**5.9–11.6**	**5.5–9.9**	**3.8–12.7**	**1.8–8.3**	**0.0–9.7**	Nature of kefir culture	0.53	
									Fermentation temperature	4.43*	
									Interaction	0.62	
Total volatiles		**99.0–178.6**	**130.8–383.6**	**146.9–256.5**	**87.7–152.2**	**102.5–315.6**	**101.4–200.6**	**4.0–41.7**	Nature of kefir culture	6.67**	
									Fermentation temperature	3.92	
									Interaction	1.32	

*$P < 0.05$, **$P < 0.01$.

acetate concentration (90 mg/L) was recorded in cider fermented by immobilized kefir culture on DCM at 5 °C. Ethyl acetate concentrations up to 150 mg/L is considered to have a positive influence (Etiévant, 1991), which was ascertained in our products, as a fruit-note was predominant.

On the other hand, low fermentation temperatures (5 °C) typically led to reduced amounts of higher alcohols, which is an important factor in cider quality, since they are considered as off flavours.

Importantly, both the increase of ethyl acetate and the decrease of higher alcohols or their percentage on total volatiles at low fermentation temperatures has been previously well documented (Herrero, Garcia & Diaz, 2006; Kourkoutas, Kanellaki, & Koutinas, 2006; Agouridis et al., 2008; Servetas et al., 2013).

Methanol is formed from methylated pectic substances (pectins) by the action of pectin esterase. The methanol content ranged <100 mg/L in all cases, which is a positive contribution, due to its toxicity effects. Of note, in traditional fermentations the usual range of methanol content is 0.1–0.2 g/L.

3.3.2. HS-SPME GC/MS analysis

For the evaluation of the aromatic profile both at ambient and high temperatures, cider samples fermented at 20 and 37 °C were analyzed using the HS-SPME GC/MS technique. Semi-quantitative results of the volatile compounds are presented in Table 5. In total, 64 compounds were detected. Esters, organic acids, alcohols, and carbonyl compounds were the most important compounds identified by the HS-SPME GC/MS technique.

From a quantitative point of view, both the fermentation temperature and the nature of kefir culture (free or immobilized) affected significantly ($P < 0.05$) the concentration of esters. On the other hand, the fermentation temperature had a significant ($P < 0.05$) effect on miscellaneous compounds, while the nature of kefir culture affected significantly total volatiles ($P < 0.05$). Noticeably, the highest ($P < 0.05$) content of esters and total volatiles was observed in cider fermented by kefir culture immobilized on apple pieces at 20 °C.

Most of the esters identified were esters of fusel alcohols, and sort chain fatty acids, known as “fruit esters”. In addition, esters of aliphatic acids like decanoic, dodecanoic, tetradecanoic, etc, mainly responsible for a yeast tone (Pisarnitskii, 2001), were also detected. Ethyl hexanoate, contributing fruity notes to wine aroma, ethyl octanoate, having a floral fruity impact, and ethyl dodecanoate which is known for its smokey, earthy, dried fruit, spicy, and toasty aroma (Miranda-Lopez, Libbey, Watson, & McDaniel, 1992) were present in all samples. A number of acetates other than ethyl acetate were also identified, such as isobutyl acetate, isoamyl acetate, 2-methylbutyl acetate, hexyl acetate, phenylmethyl acetate, 2-phenylethyl acetate, and (*Z*)-3,7-dimethyl-2,6-octadien-1-ol acetate (neryl acetate), which are known to provide a pleasant fruit-like aroma (Rapp & Mandery, 1986). In particular, isoamyl acetate, detected in all cider samples, contributes to the aromatic complexity of wines giving a banana–like aroma (Jackson, 1994). Similarly, ethyl 9-decenoate, also present in all products, adds a pleasant note (Etiévant, 1991).

Fatty acids, due to their low odor threshold values and rather high concentrations in cider, contribute to the complexity of the aroma bouquet at concentrations up to their threshold values, while at higher concentrations, their impact is negative (Jackson, 1994). A positive correlation between octanoic and decanoic acid, which were the only acids detected, and product quality has been previously reported (Etiévant, 1991).

Fusel alcohols are generally considered to have rather unpleasant odors, cotributing rather to the intensity of cider odor than to its quality (Etiévant, 1991). 2-phenylethanol, identified in all products,

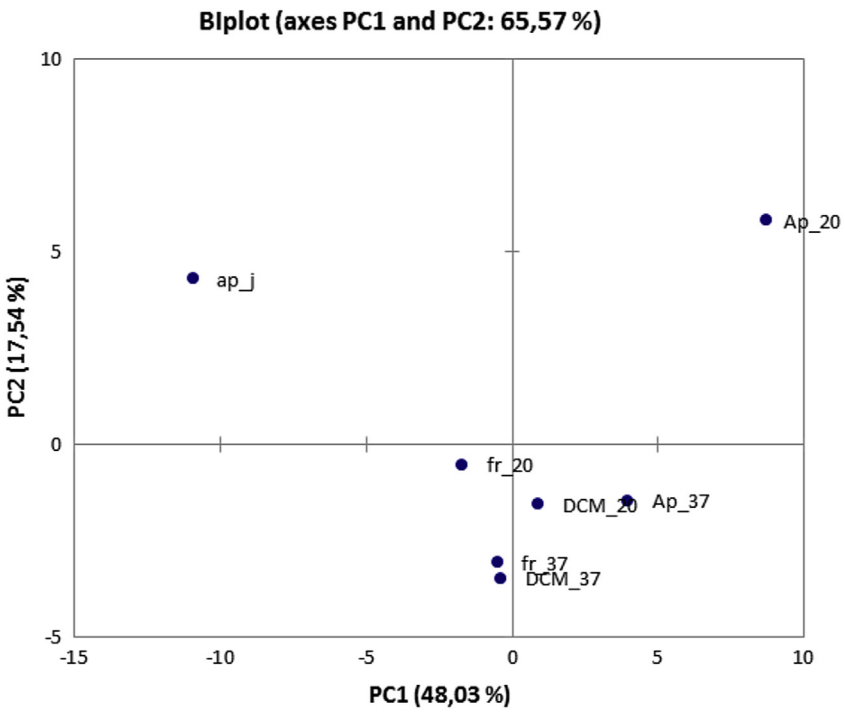

Fig. 2. PCA plot of volatiles isolated by apple juice and ciders fermented by free or immobilized kefir culture. Ap: Cider fermented by immobilized kefir culture on apple pieces, fr: Cider fermented by free kefir culture, DCM: Cider fermented by immobilized kefir culture on DCM, ap_j: Apple juice. The fermentation temperature is indicated after the sample codes.

is one of the few fusel alcohols described with pleasant odor as old rose (Etiévant, 1991).

Moreover, a number of carbonyl compounds were identified, some of which are known to contribute to the complexity of cider aroma. β-Damascenone present in some samples has a complex smell of exotic fruits (Pisarnitskii, 2001).

Of note, a few miscellaneous compounds were detected, including mainly 1,1-diethoxy-ethane (acetal) and hydrocarbons. 1,1-diethoxy-ethane has been described to provide a refreshing, fruit and green odor, although its contribution could be attributed to a decrease in acetaldehyde content, resulting thus in a smoothing and modulation of the aroma (Etiévant, 1991). Hydrocarbons are considered largely insignificant to cider aroma (Etiévant, 1991).

3.3.3. Chemometrics

The application of the PCA algorithm to HS-SPME GC/MS data showed four distinctive groups (Fig. 2). The first group consisted by apple juice, while the second group by ciders fermented by immobilized cells on apple pieces. Samples fermented by immobilized cells on DCM composed the third group and were close to ciders produced by free cells (fourth group). Ciders produced by immobilized cells on apple pieces contained compounds in higher amounts that correlated positively most to PC1, whereas low fermentation temperature (20 °C) resulted in compounds correlated most to PC2. Overall, the results showed that mainly the nature of kefir culture rather than the temperature had a significant effect on volatile composition.

3.4. Preliminary sensory evaluation

No significant differences ($P > 0.05$) in quality attributes were observed among the experimental ciders and the commercial products during the preliminary sensory investigation (Table 6). Noticeably, the new products were accepted by the panel and the fruity aroma, fine taste, and overall high quality was ascertained, despite the fact that no post fermentation treatments were carried out and ciders produced in the laboratory were poor in CO_2, which is known to contribute to freshness. Ciders produced by free and immobilized kefir culture on apple pieces had a fruity/wine-like aroma, while a yeast-like aroma was predominant in ciders produced by immobilized kefir culture on DCM.

4. Conclusions

Kefir culture proved suitable for simultaneous alcoholic and ML cider fermentation, while no species competition was noted. Cell immobilization on DCM resulted in enhanced fermentation efficiency and the recorded productivities were higher than in

Table 6
Sensory evaluation of ciders produced by free or immobilized kefir culture.

Quality attribute	Free kefir culture	Kefir immobilized on apple pieces	Kefir immobilized on DCM	Commercial cider product #1	Commercial cider product #2	*F*-values
Aroma	3.0 ± 0.5	3.1 ± 0.7	2.6 ± 0.5	3.3 ± 0.8	4.3 ± 0.8	0.85
Taste	2.5 ± 0.7	2.6 ± 0.9	2.7 ± 0.9	3.4 ± 0.8	3.9 ± 0.8	0.53
Overall quality	3.0 ± 0.7	2.6 ± 0.5	3.0 ± 0.8	4.3 ± 0.7	4.6 ± 0.5	0.49

0: unacceptable; 5: wonderful.

industrial practice. The nature of kefir culture rather than the temperature had a significant effect on volatile composition. Hence, ciders produced by immobilized cells on apple pieces contained higher amounts of esters and total volatiles. On the other hand, all new products were accepted by the panel during the preliminary organoleptic evaluation. However, more research is still required in the field, especially in issues associated with maintenance of cell viability during storage, in order to satisfy the commercial market needs and allow industrial application of the proposed technologies.

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