Development of new non-dairy beverages from Mediterranean fruit juices fermented with water kefir microorganisms

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ABSTRACT

The aim of this work was to explore the use of several Mediterranean fruit juices as fermentable substrates to develop new non-dairy fermented beverages. Microbiological, chemical and sensory features of kefir-like beverages obtained after the fermentation of juices extracted from fruits cultivated in Sicily (southern Italy) with water kefir microorganisms were investigated. Results indicated that both lactic acid bacteria and yeasts were able to develop in the fruit juices tested, but the highest levels were registered with prickly pear fruit juice. All fruit juices underwent a lactic fermentation, since a lactic acid content was detected in the resulting kefir-like beverages. Except kiwifruit and quince based kefirs, total titratable acidity increased for the other experimental products. A general decrease of the soluble solid content and an increase of the number of volatile organic compounds (VOCs) was also observed after fermentation. As expected, the fermentation increased the concentration of alcohols. The main fermentation in KLBs resulted to be yeast-based. Kiwifruit and pomegranate juices possessed a high antioxidant activity. Esters compounds were present at higher amount after the fermentation, especially in grape, pomegranate and quince. Aldehydes showed an opposite trend. Changes in colour attributes were registered as noticeable at human perception scale. The overall quality evaluation indicated that, among the Mediterranean fruit juices tested, apple and grape beverages were the products mostly appreciated by the tasters. Therefore, these findings support the possibility to develop fruit-based kefir-like beverages with high added value and functional properties.

Key words: fermentation; functional foods; kefir-like beverages; Mediterranean fruits; new fermented products

1. INTRODUCTION

Functional foods influence positively one or more biological function in the human body, improving the state of health and wellness, and reducing the risk of developing diseases (Diplock et al., 1999). This food category includes all products containing probiotic microorganisms defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Araya et al., 2002). The idea that alimentation might prevent human diseases is very old; "Let food be thy medicine and medicine be thy food" is a quote by Hippocrates 400 years B.C. (Otles and Cagindi, 2012).

Yogurt is undoubtedly the fermented milk product best known and consumed in the world. However, kefir represents another important fermented milk. It became very popular during the 20th century because researchers investigated on its contribution to better health (Shavit, 2008). Kefir was used for the treatment of tuberculosis, cancer and gastrointestinal disorders when modern medical treatments were not available and it is also associated with longevity in Caucasus, mountain region where it originated (Cevikbas et al., 1994; Zourari and Anifantakis, 1988). Nowadays, there is a renewed interest for this product (Shavit, 2008).

Water kefir is a non-dairy kefir prepared with a sucrose solution with or without fruit extracts (Schneedorf, 2012) fermented by kefir grains, consisting of a consortium of yeasts, mainly *Kluyveromyces*, *Candida* and *Saccharomyces*, and lactic acid bacteria (LAB), including the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*. All these microorganisms are embedded in a resilient water-soluble branched glucogalactan matrix named kefiran (Rodrigues et al., 2005; Gulitz et al., 2011; Magalhães et al., 2010). Several of the different bacteria and yeasts that can be found in kefir are recognized as probiotics (Latorre-García et al., 2007; de LeBlanc et al., 2006; Zhou et al., 2009a; Zhou et al., 2009b).

When grains are applied to ferment fruit juice, molasses or sugary solution, it is referred to as sugary kefir, water kefir or tibico (tibico's tepache) (Koutinas et al., 2009; Magalhães et al., 2010). Indeed, fruit juices contain water, sugar, proteins, amino acids, vitamins and minerals being a suitable and rich medium for microbial growth (Dias et al., 2003; Schwan, 1998) that can be used to prepare fermented beverages, like kefir, wine and other products (Duarte et al., 2010). Moreover, the fermentation of these substrates makes appreciated kefir beverages with acidic taste, refreshing, slightly carbonated, low alcoholic and acetic content (Grønnevik et al., 2011; Miguel et al., 2011).

Since the consumption of vegetables and fruits is strongly advised by many Governments to reduce the risk of several diseases and functional declines associated with aging (Temple, 2000; Willett, 1994; Willett, 1995), their fermentation might widen the choice for the consumption of these products. Over the years, new and diverse methods for processing fruits have been studied in an effort to minimize production losses, increasing farmers' income, and to introduce new products to the market (Duarte et al., 2010). The development of fruit juice-based fermented beverage with kefir may be perceived by consumers as healthy (Puerari et al., 2012).

Due to the numerous positive effects of kefir as well as vegetables and fruits on the human health, this work was aimed to evaluate the characteristics of kefir-like beverages obtained after the fermentation of juices extracted from fruits cultivated in Sicily (southern Italy) with water kefir microorganisms, in order to develop new non-dairy fermented beverages and to valorise the agricultural productions of this Mediterranean region.

2. MATERIALS AND METHODS

2.1. Production of fruit kefir

In this study, apple (*Malus domestica* Borkh, cv Gala), quince (*Cydonia oblonga* Mill., cv Del Portogallo), grape (*Vitis vinifera* L., white-berry cv Italia), kiwifruit (*Actinidia chinensis* Pl., cv Hayward), prickly pear (*Opuntia ficus-indica* L., cv Sanguigna) and pomegranate (*Punica granatum* L., cv Dente di cavallo) juices were subjected to fermentation. All fruits were peeled before being processed, except grape. The characteristics of the juices, just after fruit squeezing, are reported in

Tables 1-3. Fruit juices (FJ) were subjected to pasteurization at 75°C for 5 min and cooled at room temperature before processing.

Beverages were produced by backslopping: the freeze-dried microbial mixture (0.125 g) was first activated in fruit juices (50 mL) at 25°C for 72 h to develop the inoculants (Ins); each In was then added (4%, v/v) to 1 L of the corresponding juice and the fermentation was statically performed at 25 °C for 48 h.

The fermentation was carried out with a commercial water kefir microbial preparation (BioNova snc, Villanova sull'Arda, Italy) containing approximately 10^9 CFU/g of LAB (*Lactobacillus*, *Lactococcus* and *Leuconostoc*) and *Saccharomyces* spp., as declared by the producer, which were identified as *Lactobacillus fermentum* (Acc. No. KT633923), *Lactobacillus kefiri* (Acc. No. KT633919), *Lactococcus lactis* (Acc. No. KT633921), *Leuconostoc mesenteroides* (Acc. No. KT633927) and *Saccharomyces cerevisiae* (Acc. No. KT724951) by Corona et al. (under review). Kefir-like beverage (KLB) productions were carried out in triplicate.

It should be emphasized that such an extensive 72 h fermentation period, designed to simulate backslopping, can result in strain ratios different from that of the originating freeze-dried starter. Thus, one would expect in the Ins a selective survival/growth of the acid-resistant strains, particularly the yeasts.

2.2. Microbiological analyses

FJs, Ins and KLBs were microbiologically investigated for several microbial populations. Decimal dilutions of samples, subjected to agitation by means of an orbital shaker (150 rpm for 1 min), were prepared in Ringer's solution (Sigma-Aldrich, Milan, Italy). Since no high-shear homogenization of the sample was carried out in order to break cell chains of lactic acid bacteria, the CFUs might be slightly underestimated (Champagne et al., 2011). Cell suspensions were plated and incubated as follows: total mesophilic count (TMC) spread plated on plate count agar (PCA), incubated aerobically at 30°C for 72 h; *Enterobacteriaceae* pour plated on double-layered violet red bile glucose agar (VRBGA), incubated aerobically at 37°C for 24 h; pseudomonads spread plated on *Pseudomonas* agar base (PAB) supplemented with 10 mg/mL cetrimide fucidin, incubated aerobically at 20°C for 48 h; rod LAB pour plated on de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 mol/L) and incubated anaerobically at 30°C for 48 h; coccus LAB pour plated on M17 agar, incubated anaerobically at 30°C for 48 h; yeasts spread plated on dichloran rose Bengal chloramphenicol (DRBC) agar, incubated aerobically at 25°C for 48 h. Count plates were carried out in duplicate for each independent production.

2.3. Monitoring of dominant strains

LAB and yeast colonies (almost four for each morphology observed) developed on the agar media from the highest dilutions of the cell suspensions of the freeze-dried commercial starter preparation and KLBs were isolated, purified to homogeneity by successive sub-culturing on the same agar media used for plate counts, checked microscopically, transferred in broth media and subjected to strain differentiation.

DNA from broth cultures, developed overnight at the optimal temperatures, was extracted by InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instruction and used for PCRs. The differentiation of the bacterial isolates was performed by random amplification of polymorphic DNA (RAPD)-PCR analysis. Strain typing was carried out in 25-µL reaction mix using the single primers M13, AB111, and AB106 as previously described by Settanni et al. (2012). Yeasts were subjected to the interdelta sequence analysis (ISA), as described by Legras and Karst (2003).

The PCR products and the molecular size marker GeneRuler 100 base pair (bp) Plus DNA ladder (M Medical Srl, Milan, Italy) were separated by electrophoresis on 1.5% (w/v) agarose (Gibco BRL, Cergy Pontoise, France) gels. The gels were stained with the SYBR® safe DNA gel stain

(Molecular probes, Eugene, OR, USA) and visualised by UV trans-illumination. The polymorphic profiles were analyzed using Gelcompare II software version 6.5 (Applied-Maths, Sint-Marten-Latem, Belgium). The monitoring of the dominant strains after fermentation was obtained by profile comparison.

2.4. Physico-chemical determinations

FJ and KLB samples were subjected to several determinations. Analyses of pH and soluble solids were performed according to the methodology reported by the Association of Official Analytical Chemistry (AOAC, 2000). Measurements of pH were determined electrometrically using the pH meter BASIC 20+ (Crison Instrument S.A., Barcelona, Spain). Soluble solid content (SSC) was measured using a digital refractometer (MTD-045nD, Three-In-One Enterprises CO. Ltd., Taiwan) and reported as °Brix. Total titratable acidity (TTA) was determined by titration of the samples with 0.1 N NaOH to an end point of pH 8.1 and expressed as g/L of citric acid. Total phenolic compounds (TPs) were analysed according to the Folin-Ciocalteu procedure (Slinkard and Singleton, 1977) and the results were expressed as mg/L of gallic acid equivalent (GAE). The antioxidant activity was determined as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (%) as described by Larrauri et al. (1998). Aliquots of 0.1 mL of each sample were added to 2.9 mL DPPH (Sigma–Aldrich Co., Milan, Italy) methanol solution (120 µmol/L). The absorbance was measured at 517 nm after an incubation step at 30 °C for 30 min in the dark. DPPH methanol solution was used as control. The results were calculated as follows: DPPH radical scavenging activity (%) = 100 – (absorbance of sample/absorbance of control) x 100.

The total anthocyanins content (TAC) were determined according to Fuleki and Francis (1968) with some modifications (Lee et al., 2005). Two different dilutions of each sample were prepared using potassium chloride buffer (0.0025 M) for pH 1.0 and sodium acetate buffer (0.4 M) for pH 4.5. Samples were diluted to a final volume of 5 mL (dilution factor = 5). The absorbance of the dilutions (pH 1.0 and pH 4.5) were achieved spectrophotometrically with the Beckman DU640 UV–vis Spectrometer (Minnesota, USA) at both 520 and 700 nm versus a blank of distilled water. This 700 nm wavelength reading was performed to correct the calculations taking into account the haze of FJs and KLBs (Lee et al., 2005). TAC (mg/L), expressed as Cyanidin-3-glucoside (Cy-3-glc) equivalents, was calculated according to the following formula:

$$TAC = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

where A = ($A_{520\ nm}$ – $A_{700\ nm}$)pH 1.0 – ($A_{520\ nm}$ – $A_{700\ nm}$)pH 4.5; MW (molecular weight) = 449.2 g/mol for Cy-3-glc; DF = dilution factor (5); l = path length in cm; ε = 26900 molar extinction coefficient for Cy-3-glc, and 10^3 = factor for conversion from g to mg.

Ethanol, acetic and lactic acids were spectrophotometrically detected for each compound (Boehringer Mannheim/R-Biopharm; Darmstadt, Germany) and applying the UV-method specified by the supplier for each determination. UV-measurements were carried out with the spectrophotometer reported above.

Carbon dioxide was indirectly determined by measuring the weight loss before and after the fermentation and expressed as g/100 mL (Zilio et al., 2004).

Colour of FJ and KLB samples were measured with a colorimeter (Chroma Meter CR-400, Minolta, Osaka, Japan), recording CIElab chromaticity coordinates (L*, a*, b*), where L* is the lightness, a* and b* are color-opponent dimensions, redness and yellowness, respectively. Chroma (C*), Hue angle (h°) and color differences (ΔE) parameters were indirectly calculated as follow: $C^* = (a^{*2} + b^{*2})^{1/2}$; h° = arctan(b*/a*) when a* > 0 and b* > 0, or as h° = 180° + arctan(b*/a*) when a* < 0 and b > 0 or as h° = 360° + arctan(b*/a*) when a* > 0 and b < 0 (McLellan *et al.*, 1995); $\Delta E = [(L_{KLB}^* - L_{FJ}) + (a_{KLB}^* - a_{FJ}) + (b_{KLB}^* - b_{FJ})]^{1/2}$ (CIE, 1995), where L_{KLB}^*, a_{KLB}^* and b_{KLB}^* are the values of KLBs, while L_{FJ}, a_{FJ} and b_{FJ} are referred to FJs. All chemicals were purchased from

WWR International (Milan, Italy), except when differently reported . Five readings were performed for each replicate of each sample.

2.5. Determination of volatile organic compounds (VOCs)

FJs and KLBs were also subjected to GC/MS analysis in order to identify the volatile organic compounds (VOCs). The extractions of VOCs were carried out using a SPME fiber of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; Supelco, Bellefonte, PA). Before being injected into the GC/MS apparatus, the fiber was subjected to an exposure step (30 min at 40 °C) to the headspace of the sample vial (10 mL of sample added with 0.5 g of NaCl) inserting it through the septum. Vials were 20 mL of volume, clear with screw top and hole caps with PTFE/silicone septa 27136 (Supelco, Bellefonte, PA). The fiber used was conditioned at 250 °C for 30 min in the GC/MS injector before extraction. 150µL of 1-Heptanol solution (35mg/L 1-heptanol in 20% ethanol aqueous solution) was used as an internal standard.

The SPME fibre was directly inserted into a Finnegan Trace MS for GC/MS (Agilent 6890 Series GC system, Agilent 5973 Net Work Mass Selective Detector; Milan, Italy) equipped with a DB-WAX capillary column (Agilent Technologies; 30 m, 0.250 mm i.d., film thickness 0.25 µm, part no 122-7032). The GC-MS conditions were those reported by Corona (2010). Individual peaks were identified by comparing their retention indices to those of control samples and by comparing their mass spectra with those within the NIST/EPA/NIH Mass Spectral Library database (Version 2.0d, build 2005). Volatile compounds were expressed as µg/L. VOC determinations were performed in triplicate for each sample.

2.6. Sensory quality

The fermented beverages were subjected to the overall quality assessment by fifteen untrained tasters (6 females and 9 males, 14 Italians and one Turkish, 25–37 years old). The samples were kept at 10 °C and aliquots of 10 mL were served, in a randomized order, in transparent glasses (50 mL volume) covered with Petri dishes and marked with three digit random numbers. Before evaluation, the tasters ate a plain biscuit and drank cold, filtered tap water. A water kefir, produced with the same starter preparation, was used as control to compare the fruit kefir beverages. The overall quality was evaluated on a 9-point hedonic scale (9= extremely pleasant; 1= extremely unpleasant). Three samples were tested in each session and the evaluation of each product was carried out in triplicate (Muir et al., 1999).

2.7. Statistical and explorative multivariate analysis

Microbiological, chemical and sensory data were analyzed using a generalized linear model (GLM). The post-hoc Tukey's method ($P<0.05$) was used to determine differences among the overall quality of KLBs. Statistical data processing was achieved by using STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA).

Microbiological (PCA, VRBGA, PAB, MRS, M17, DRBC counts of KLBs and the differences in counts between unfermented and fermented products), chemical (pH, TTA, SSC, TP, DPPH, ΔE, ethanol, lactic acid, acetic acid and CO_2), VOC (grouped as acids, alcohols, aldehydes, functional groups, esters, ketones, phenols, aromatic hydrocarbons and terpenes) and sensory attributes were subjected to an explorative multivariate analysis to investigate relationship among data obtained from the different experimentations (Rodríguez-Gómez et al., 2012). The principal component analysis (PCAn) explored the input matrix based on the normalized average data of the three replicates, preliminary evaluated by using the Barlett's sphericity test (Dillon and Goldstein, 1984; Mazzei *et al.*, 2010).

Only factors resulted to have eigen-values higher than 1.00 were selected according to the Kaiser criterion (Jolliffe, 1986). Statistical data processing and graphic construction were achieved by using STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA) and XLStat software version 2015.1.1 (Addinsoft, New York, USA).

3. RESULTS

3.1. Microbiological characteristics of fruit juices and fermented beverages

The microbial communities characterizing FJs, before pasteurization, Inoculants and KLBs are given in Table 1. Except prickly pear FJ, which showed the presence of all six microbial populations investigated, the other FJs displayed better hygienic conditions. In particular, none of the six microbial groups was found at detectable levels for grape, kiwifruit and pomegranate FJs, while low levels of TMC, coccus-shaped LAB and yeasts were registered for apple and quince FJs. The thermal treatment reduced all microbial groups at levels below the detection limits (results not shown).

The inoculants developed with the commercial kefir starter preparation were almost all characterized by 10^7 CFU/mL of TMC, LAB rods and cocci and yeasts, with the exception of pomegranate In showing a slightly lower level of LAB rods. *Enterobacteriaceae* and pseudomonads were only detected for prickly pear In.

After addition of Ins to the final volumes of FJs to be transformed into KLBs, all microbial groups resulted diluted of almost 2 orders of magnitude. At the end of fermentation, KLBs were characterized by almost the same levels of microorganisms as those registered for Ins, even though LAB rods in pomegranate KLB were ten folds higher than the corresponding In, as well as both LAB groups and TMC in prickly pear KLB. *Enterobacteriaceae* and pseudomonads were detected only in prickly pear KLB.

Four different LAB strains (one for each species) were found at 10^9 CFU/g in the commercial starter preparation. At the same concentration, the freeze-dried culture contained only one *S. cerevisiae* strain (Fig. 1). The direct comparison of the genomic patterns (results not shown) allowed the recognition and monitoring of the added cultures after fermentation of the FJs, confirming their dominance during the transformation process.

3.2. Physico-chemical analyses

Chemical determinations are shown in Table 2. Except prickly pear FJ which was characterized by an initial pH above 6, all other FJs showed pH values below 4. KLBs generally showed pH values slightly higher than the corresponding FJs, especially kiwifruit and quince KLB, with the exception of prickly pear KLB for which a decrease of 2.15 units was registered.

TTA for the couples FJ/KLB was highly different among samples. In particular, the lowest TTA value was found for prickly pear FJ (0.38 g/L citric acid). TTA increased after fermentation for apple, grape, pomegranate and prickly pear KLBs, with the last product showing the highest increase. TTA decreased for kiwifruit and quince KLBs. However, the last two unprocessed FJs were characterized by high TTA values (13.53 and 9.11 g/L citric acid, respectively).

SSC of FJs ranged between 11.67 and 15.73 °Brix. All SSCs decreased after the fermentation: the highest reductions were registered for grape. On the contrary, a decrease of only 1.77 °Brix was recorded for kiwifruit KLB.

FJs TP was highly variable, ranging from 131.61 mg/L in grape to 1325.20 mg/L in pomegranate samples. All KLBs showed lower levels of TP than the corresponding FJs, with the highest decrease (53%) displayed by grape KLB. Barely 10% of TP reduction was recorded for kiwifruit and quince KLBs.

DPPH results showed that kiwifruit and pomegranate FJs possessed a high antioxidant activity, 94.70 and 91.93%, respectively. Grape FJ had only 34.21% of antioxidant activity which decreased of 19.08% after fermentation. The other DPPH decreases were between 3.34 and 5.19%. Anthocyans (mg/L of Cy-3-glc equivalent) have been detected only in pomegranate FJ and KLB. The last samples underwent a reduction of TAC of 57%.

Regarding colour parameters, Lightness (L*) generally increased after fermentation. Redness (a*) reduced significantly for prickly pear and pomegranate KLBs, whereas increased for grape KLB. Yellowness (b*) and chroma (C*) values were not significantly different for FJs and the corresponding KLBs, with the exception of pomegranate that showed an increase of the blue component and a decrease of the saturation after fermentation. The variation of hue angle was different among the samples. The hue angle of apple and kiwifruit KLBs decreased, while pomegranate, prickly pear and quince showed a significant increase. ΔE ranged between 3.41 (kiwifruit) and 14.91 (prickly pear).

KLBs were also analyzed for ethanol, acetic and lactic acids and the results are reported in Table 2. Ethanol content ranged between 1.03 and 4.96% (v/v), with pomegranate, quince and grape KLBs showing the highest values. The fermentation of kiwifruit FJ generated only 1.03% (v/v) of ethanol. The lowest amount of lactic acid (0.02 g/L) was detected for apple and grape KLBs, while the highest value (1.00 g/L) was recorded for prickly pear KLB. Acetic acid was below 0.10 g/L for apple and pomegranate KLBs, while levels between 0.11 and 0.16 g/L were found for the other KLBs.

3.3. Volatile organic compounds (VOCs)

The composition of the VOCs of FJs and KLBs is shown in Table 3. A total of 107 different compounds belonging to acids, alcohols, aldehydes, esters, ketones, phenols, sulphur compounds, aromatic hydrocarbons and terpenes were detected.

In general, a significant increase of the number and percentage of VOCs was obtained after fermentation. In particular, acids increased for grape (mainly hexanoic acid), quince and pomegranate KLBs. As expected, the fermentation increased the alcohols. Diol 2,3-butanediol was detected in all samples; it is produced by LAB via the butanediol fermentation pathway. 1-hexanol increased during fermentation in all KLBs except pomegranate and grape beverages. Grape, kiwifruit, prickly pear and quince KLBs showed a consistent increase of isoamylalcohol and phenylethylalcohol. Ester compounds showed a higher amount after the fermentation, especially in grape, pomegranate and quince. Only aldehydes decreased after fermentation.

3.4. Overall quality assessment

The results of the overall quality of the KLBs evaluated by the 15 untrained tasters are graphically reported in Fig. 2. Compared to the water kefir, prepared with the same starter culture, grape and apple KLBs gained the highest overall quality evaluation, while the product resulting from the fermentation of quince juice was less appreciated.

3.5. Statistical and explorative multivariate analysis

A study considering several parameters simultaneously is of interest for a general evaluation of the different products obtained in this study. Indeed, the multivariate elaboration has been widely applied in food processes (Berrueta et al., 2007). The PCAn performed with microbiological, chemical and sensory data led to the identification of Factors explaining the total variance.

Regarding the microbial loads, the correlation analysis among variables (Tab. S1) showed that there were many significant relationships among them and the data were found to be appropriated to be subjected to the PCAn in order to condense the information with Factors.

Microbial loads and pH changes exhibited that the first three Factors gained eigenvalues higher than 1. The discrimination of samples is reported in the biplot of Fig. 3 showing the projection of the cases (KLBs) onto the planes as a function of Factors 1 and 2. The first two Factors represented up to 83.12% of the total variance. Factor 1 manly contributed to discriminate cases, in fact, all samples resulted closely related to each other with the exception of prickly pear KLB, that was positively correlated with both Factors. Prickly pear KLB, indeed, showed microbial counts on average higher than others, in particular referred to PAB and VRBGA, and as well as higher pH values. On the other hand, the variables associated to the Factor 2 contributed only marginally to discriminate samples; in particular, kiwifruit and grape KLBs showed the greatest variance in terms of ΔTMC and ΔMRS.

The discrimination of samples based on PCAn of chemical variables (Fig. 4) highlighted differences among samples that resulted widely spread in the bi-plot. Four Factors displayed eigenvalues higher than 1 and the first two Factors explained 62.62% of the total variance. Factor 1, representing 37.01% of the total variance (positively correlated with TTA, TP, DPPH and negatively with pH and ΔE, as reported in Tab. S2), displayed a continuous variance, while Factor 2 clearly distinguished kiwifruit and prickly pear KLBs from the other products. Since Factor 2 is mainly correlated with SSC, ethanol and lactic acid.

Regarding VOCs (Fig. 5) a total of three Factors (accounting for 50.96, 26.60 and 14.42% of total variance) showing eigenvalue higher than 1.00 were found. The Factor 1 and Factor 2 explained 50.96 and 26.59% of total variance, respectively. The descriptors that mainly contributed to the Factors are reported in Table S3. Based on the bi-plot, grape KLB resulted broadly different from the other samples, displaying a positive correlation with the amount of acids, esters and terpenes. Apple and pomegranate resulted grouped together on the lower-left quarter, due to their increase in phenols. Factor 2 distinguished quince, prickly pear and kiwifruit KLBs, mainly for the higher production of aldehydes and ketones than other samples.

4. DISCUSSION

In this work, a water kefir microbial preparation containing *Saccharomyces* spp. and different LAB was applied to ferment fruit juices to produce kefir-like products. In order to provide enough volume of each FJ stable over time and with the same microbiological characteristics for the entire experimentation, FJ bulks were pasteurized soon after squeezing.

In general, freeze-dried starter cultures containing LAB and yeasts are used for food fermentations carried out at industrial level (Güzel-Seydim et al., 2010; Mistry, 2004). Regarding kefir production, the activity of the microbial populations are affected by the quality of kefir grains, the ratio between grains and substrate, duration and temperature of incubation, sanitation conditions and storage (Güzel-Seydim et al., 2011; Altay et al., 2013). Moreover, several interactions can determine an increase or, on the contrary, a decrease of the number of kefir microorganisms (Sieuwerts et al., 2008; Nambou et al., 2014). In our study, a general decrease in concentration was estimated both for LAB and yeasts immediately after inoculation. This observation might be explained by a loss of viability of many of the cultures, probably as a consequence of the too acidic conditions of most of the FJs.

As previously reported (Chen et al., 2008), two groups of microorganisms co-exist in kefir products: lactic acid bacteria and yeasts. In our experiment, LAB and TMC reached similar amount of those detected in other sugary kefirs (Sabokbar and Khodaiyan, 2014; Liu and Lin 2000), with no significant differences between cocci and bacilli (Magalhães et al., 2010). The same findings were achieved by Irigoyen et al. (2005) on milk-based kefir after two days, since lactobacilli and lactococci counts were 10^8 cfu/ml. On the contrary, significant fewer counts were reported by Koroleva (1982) for lactobacilli and by Kilic et al. (1999) for lactococci, but, in any case, the counts of LAB rods and cocci followed the same pattern. Babina and Rozhokova (1973) found that lactobacilli of kefir grains increased viscosity and thus enhanced the consistency of kefir. On the contrary, yeast population was about 2 log CFU/mL higher than detected by Sabokbar and Khodaiyan (2014), Liu and Lin (2000) and Rosi (1978), and in line with the level reported by Kilic et al. (1999). Prickly pear FJ was characterized by the highest microbial loads. Although after pasteurization *Enterobacteriaceae* and pseudomonads were undetectable, they developed after fermentation. This result might be imputable to the almost neutral pH (6.26) of prickly pear juice that is not inhibitory for these undesired microorganisms.

The persistence of the starter strains during fermentation was monitored by strain typing and comparison of the genomic patterns. Specifically, the isolates were collected from a given medium at the highest dilutions of KLBs and, after PCR, the polymorphic profiles were compared with those of the strains isolated from the freeze-dried culture. This approach allowed the recognition and monitoring of the added cultures and confirmed that the highest levels estimated on a given medium was due to the inoculated strains.

Microbiological and chemical evaluations indicated that the fruit juices behaved differently in presence of the microorganisms inoculated. Except kiwifruit KLB, all other products are classified as alcoholic beverages according to the Italian legislation (GURI 90, 2001), since ethanol content was higher than 1.2% (v/v). A strict correlation was found between the decrease of SSC and the increase of ethanol and CO_2 formation for all samples. However, a consistent reduction of SSC was not observed for kiwifruit FJ, probably because the low initial pH slowed down the development of LAB and yeast.

S. cerevisiae, which exhibits strong fermentative metabolism and tolerance to ethanol, is primarily responsible for alcohol production and it has been previously identified in kefir like beverages (Pereira et al., 2010). The end products of yeast fermentation, ethanol and CO_2, are critical in producing the exotic flavor and yeasty aroma of authentic kefir (Güzel-Seydim et al., 2000a,b). Some species within the genus *Lactobacillus* have also the ability to produce ethanol, since they have alcohol dehydrogenase activity, an enzyme able to convert acetaldehyde to ethanol (Magalhães, et al., 2011a; Magalhães, et al., 2011b; Puerari et al., 2012).

Beshkova et al. (2003) reported that alcohol content should be enough to give kefir a typical light alcoholic flavor. However several studies showed low ethanol levels in kefir beverages using different substrates (Magalhães, et al., 2011a; Magalhães, et al., 2011b; Zajšek, K., & Goršek, 2010). In our study, limited levels of ethanol were estimated after 48 h of fermentation. The residual SSC detected at the end of the process might suggest a partial fermentations of FJs.

Kiwifruit and quince KLBs showed a decrease of TTA in comparison with the corresponding FJs. The high TTA observed in kiwi and quince FJs was mainly due to malic and quinic acid, as well as citric acid as reported by Schäfer et al., (1996) and Silva et al., (2004). The decrease is explained by the consumption of organic acids during the fermentation process at 25 °C (Puerari et al., 2012). Furthermore, the ability to ferment and assimilate the organic acids, as carbon and energy sources, causes an increase of pH value (Lopandic et al., 2006). However, the increase of TTA was registered in some KLBs. Since an increase in pH and TTA has been observed in some fruits during the storage under different thermal regimes (da Silva et al., 2013), it might be supposed that the results displayed by apple, grape and pomegranate KLBs are imputable to the extraction of organic acids from the residual part of pulp still present in the juices during fermentation.

Prickly pear FJ underwent a lactic fermentation since a high lactic acid content was detected in the resulting KLB and also because of the production of acetic acid (heterolactic fermentation). For this KLB, a moderate amount of ethanol was registered after fermentation, probably due to the quicker development of LAB over yeasts. Except for prickly pear KLB, lactic and acetic acid levels registered in this work are quite low to significantly affect the sensory properties of the final products. Furthermore, the ethanol levels were generally in line with the reduction of soluble solid content; thus, the main fermentation in KLBs appears to have been yeast-based.

The total content of polyphenols was positively correlated to the antioxidant activity before and after fermentation. High values of polyphenols generally determine high antioxidant activity (Dani et al., 2007), but this phenomenon may depend on fruit maturity and cultivation practices (Burin et al., 2010). The radical scavenging activity is positively associated to a high content in anthocyans, as registered for pomegranate juice (Gil et al., 2000).

Regarding color parameters, the reduction of lightness and redness in KLBs could be explained by the browning processes occurring during fermentation. This phenomenon is due to the activation of certain oxidases, such as polyphenol oxidase, when the environments are not completely anaerobic

(Corona, 2010). Considering the just noticeable differences limit of 2.3 on a human perception scale reported by Mahy *et al.* (1994), all the samples changed their colors (ΔE) after the fermentation process. The most noticeable changes were for prickly pear, pomegranate and grape KLBs.

Based on VOC determination, the higher aromatic complexity of the final products, compared to the FJs, was evidenced by the higher number of molecules recognized. Volatile compounds determine different desirable sensory characteristics contributing to the aroma of beverages (Arrizon et al., 2006). The alcohols are reported to be particularly important for the flavour of dairy fermented beverages (Athanasiadis et al., 2001; Dragone et al., 2009; Magalhães et al., 2011a).

Propionic acid, an important odor-active compound, was mainly detected in pomegranate KLB. This compound shows antimicrobial properties (Nualkaekul and Charalampopoulos, 2011) and could be important for the biopreservation of the transformed product. However, in this kind of product, the control of the growth of food spoilage microorganisms can also be attributed to the organic acids produced by yeasts and bacteria (Settanni and Moschetti 2010). These compounds might be defining also for the sensory evaluation of the fermented product carrying on a refreshing flavor, unique aroma and texture (Duarte et al., 2010). Moreover, esters compounds are largely responsible for the fruity aroma associated with kefir yeast cultures (Nambou et al., 2014). Glycerol, the main secondary product in alcoholic fermentations led by *S. cerevisiae* (Puerari et al., 2012), was detected in all KLB, but at concentrations too low to confer body and texture to KLBs (Dias et al., 2007). Apple and grape KLBs gained the highest scores at the overall quality evaluation.

The results of this study showed that processing Mediterranean fruit juices with water kefir microorganisms determined the production of some beverages, in particular apple and grape KLBs, with high added value and appreciated by testers.

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Table 1. Microbial loads (Log CFU/mL) of fruit kefir-like beverages.

Sample	Media					
	PCA	VRBGA	PAB	MRS	M17	DRBC
Unpasteurized fruit juices						
Apple	1.7±0.2	0	<1	0	1.9±0.9	1.3±0.1
Grape	0	0	<1	0	0	0
Kiwifruit	0	0	<1	0	0	0
Pomegranate	0	0	<1	0	0	0
Prikly pear	5.5±0.2	1.5±0.2	1.7±0.4	1.8±0.6	4.5±0.2	2.0±0.4
Quince	2.5±0.4	0	<1	0	0.3±0.1	0
Inoculants						
Apple	7.2±0.3	0	<1	7.5±0.1	7.2±0.3	7.5±0.5
Grape	7.5±0.3	0	<1	7.5±0.2	7.2±0.5	7.2±0.7
Kiwifruit	7.4±0.5	0	<1	7.5±0.6	7.0±0.8	7.0±0.5
Pomegranate	7.8±0.4	0	<1	6.7±0.3	7.1±0.6	7.5±0.3
Prikly pear	7.2±0.3	3.9±0.4	4.1±0.4	7.3±0.8	7.2±0.4	7.4±0.3
Quince	7.7±0.9	0	<1	7.5±0.3	7.1±0.1	7.3±0.7
Non-fermented KLBs						
Apple	5.2±0.7	0	<1	5.3±0.4	5.1±0.3	5.2±0.6
Grape	5.7±0.4	0	<1	5.9±0.8	5.9±0.8	5.2±0.8
Kiwifruit	4.3±0.4	0	<1	4.7±0.1	4.7±0.6	5.2±0.7
Pomegranate	5.3±0.4	0	<1	5.6±0.9	4.9±0.2	5.5±0.6
Prikly pear	5.2±0.9	1.7±0.3	1.9±0.4	5.8±0.8	5.3±0.9	6.2±0.8
Quince	5.4±0.9	0	<1	5.3±0.9	4.3±0.4	5.3±0.4
Fermented KLBs						
Apple	7.5±0.7	0	<1	7.7±0.3	7.4±0.8	7.4±0.2
Grape	7.9±0.3	0	<1	7.9±0.3	8.0±0.1	7.9±0.6
Kiwifruit	7.4±0.8	0	<1	7.6±0.8	6.6±0.1	7.6±0.7
Pomegranate	7.9±0.5	0	<1	7.7±0.6	7.5±0.4	8.0±0.9
Prikly pear	8.4±0.4	4.0±0.9	4.9±0.5	8.0±0.2	8.3±0.7	7.6±0.5
Quince	7.8±0.3	0	<1	7.7±0.8	7.6±0.1	7.8±0.5

Results represent mean values ± SD of six measurements (carried out in duplicate for three independent productions). Abbreviations: PCA, plate count agar for total mesophilic counts; VRBGA, violet red bile glucose agar for *Enterobacteriaceae*; PAB, *Pseudomonas* agar base for pseudomonads; MRS, de Man-Rogosa-Sharpe agar for rod LAB; M17, medium 17 agar for mesophilic coccus LAB; DRBC, dichloran rose Bengal chloramphenicol agar for yeasts; KLB, kefir-like beverage.

Table 2. Physico-chemical analysis of fruit juices and kefir-like beverages.

Sample		pH	Ethanol (% v/v)	Lactic acid (g/L)	Acetic acid (g/L)	CO_2 (g/100 mL)	TTA (g/L citric acid)	SSC (°Brix)	TP (mg/L)	DPPH (%)	TAC (mg/L Cy-3-glc)	Color					
												L*	a*	b*	C*	H°	ΔE
Apple	FJ	3.70±0.06	n.d.	n.d.	n.d.	n.d.	1.88±0.04	12.03±0.11	203.90±1.21	41.19±0.34	n.d.	39.45±0.11	5.54±0.07	14.70±0.06	15.72±0.14	69.23±1.30	
	KLB	4.04±0.08***	2.67±0.14	0.02±0.00	0.06±0.01	1.51±0.25	2.35±0.02***	8.70±0.13**	176.40±1.57 ns	37.56±0.27 ns	n.d.	38.31±0.14 ns	6.02±0.06 ns	12.79±0.08 ns	14.17±0.21 ns	62.87±1.47*	5.06±1.04
Grape	FJ	3.61±0.11	n.d.	n.d.	n.d.	n.d.	2.66±0.06	14.93±0.09	131.61±1.67	34.21±0.41	n.d.	34.69±0.10	3.98±0.11	8.34±0.05	9.25±0.13	64.49±1.21	
	KLB	3.81±0.04**	4.44±0.24	0.02±0.01	0.16±0.03	1.83±0.49	2.91±0.05***	8.47±0.08***	61.96±1.34**	15.13±0.23**	n.d.	40.46±0.15*	5.39±0.12*	14.22±0.11 ns	15.30±0.15 ns	66.71±1.65 ns	9.06±1.58
Kiwifruit	FJ	3.06±0.13	n.d.	n.d.	n.d.	n.d.	13.53±0.09	11.73±0.06	938.58±1.89	94.70±0.27	n.d.	40.39±0.13	-4.70±0.07	14.38±0.13	15.13±0.09	108.09±1.74	
	KLB	3.48±0.03**	1.03±0.09	0.13±0.03	0.11±0.02	0.90±0.17	12.81±0.10***	9.97±0.08 ns	843.42±2.14 *	89.51±0.15 ns	n.d.	41.85±0.12 ns	-4.28±0.13 ns	16.47±0.15 ns	17.02±0.05 ns	104.70±1.96 ***	3.41±0.73
Pomegranate	FJ	3.66±0.09	n.d.	n.d.	n.d.	n.d.	4.07±0.06	15.73±0.07	1325.20±1.45	91.93±0.38	132.44±1.42	22.26±0.11	14.45±0.08	-7.46±0.09	16.26±0.17	332.68±1.11	
	KLB	3.89±0.08**	4.96±0.30	0.05±0.00	0.07±0.01	3.21±0.55	4.29±0.01***	9.37±0.14***	898.70±1.17**	88.04±0.43 ns	56.40±0.33**	30.18±0.13***	12.19±0.09**	-3.25±0.04***	12.65±0.12***	345.04±1.23***	9.35±1.62
Prickly pear	FJ	6.26±0.16	n.d.	n.d.	n.d.	n.d.	0.38±0.03	14.07±0.15	546.64±1.93	62.99±0.35	n.d.	27.05±0.08	21.08±0.13	10.30±0.14	23.47±0.27	25.98±1.17	
	KLB	4.11±0.07***	2.31±0.19	1.00±0.10	0.16±0.02	1.88±0.34	1.92±0.07***	9.67±0.11**	374.13±0.98*	59.65±0.15***	n.d.	32.93±0.07**	10.57±0.14***	16.51±0.17 ns	20.06±0.18 ns	55.34±1.25***	14.91±2.69
Quince	FJ	3.19±0.03	n.d.	n.d.	n.d.	n.d.	9.11±0.12	11.67±0.07	359.16±1.73	60.36±0.27	n.d.	40.00±0.10	5.25±0.09	12.25±0.16	13.33±0.19	66.81±1.31	
	KLB	3.62±0.05**	4.51±0.31	0.18±0.01	0.11±0.01	2.42±0.21	7.43±0.05***	5.87±0.10**	322.71±1.62 ns	60.53±0.16*	n.d.	44.21±0.16***	5.26±0.11 ns	13.85±0.05 ns	14.82±0.23 ns	69.10±1.07**	4.87±1.20

Mean values ± SD of five measurements for each replicate.
Abbreviations: FJ, fruit juice; KLB, kefir-like beverage; CO_2, carbon dioxide; TTA, total titratable acidity; SSC, soluble solid content; TP, total phenol (gallic acid equivalent mg/L); DPPH, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (%); TAC, total anthocyanin content (mg/L Cyanidin-3-glucoside equivalents); L*, lightness; a*, redness; b*, yellowness; H°, hue angle; ΔE, color differences; n.d., not detectable.
P value: ***, p≤0.001, **, p≤0.01; *, p≤0.05; ns, not significant. Significant differences among fruit juices and fermented kefir-like beverages for each fruit sample and each physico-chemical determination ($p < 0.05$).

Table 3. Analysis of the volatile organic compounds emitted from fruit juices and kefir-like beverages.

Chemical compound	Apple		Grape		Kiwifruit		Pomegranate		Prickly Pear		Quince	
(μg/L)	FJ	KLB	FJ	KLB	FJ	KLB	FJ	KLB	FJ	KLB	FJ	KLB
Acids												
Acetic acid	3.34	26.62	5.80	142.98	7.80	162.62	12.86	269.83	3.72	133.95	59.77	268.80
Propionic acid	n.d.	0.83	n.d.	n.d.	n.d.	n.d.	n.d.	4.97	n.d.	n.d.	n.d.	n.d.
Isobutyric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	47.42
Hexanoic acid	n.d.	n.d.	n.d.	4227.56	n.d.	308.74	n.d.	174.10	n.d.	52.63	n.d.	295.18
Eptanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.12
Octanoic acid	n.d.	59.39	n.d.	136.14	n.d.	173.24	n.d.	330.10	n.d.	69.20	n.d.	382.33
Decanoic acid	n.d.	25.34	n.d.	20.38	n.d.	32.33	n.d.	68.00	n.d.	14.37	n.d.	103.40
Hexadecanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.32
Total	3.34	114.10	5.80	4527.06	7.80	676.93	12.86	847.01	3.72	270.15	60.90	1117.56
Alcohols												
Isobutanol	n.d.	n.d.	n.d.	68.32	n.d.	428.76	n.d.	127.05	n.d.	9.22	n.d.	198.78
Isoamylalcohol	n.d.	408.78	n.d.	3579.72	n.d.	3729.22	n.d.	n.d.	n.d.	1540.98	n.d.	1902.59
1-pentanol	1.07	n.d.	n.d.	n.d.	3.47	10.20	n.d.	n.d.	45.80	n.d.	n.d.	n.d.
1-hexanol	73.91	102.62	105.13	218.38	123.00	n.d.	41.18	18.43	559.18	1064.34	345.88	637.64
cis-3-hexen-1-ol	n.d.	n.d.	0.59	n.d.	n.d.	13.23	21.67	14.91	32.00	n.d.	68.70	69.95
trans-2-hexenol	n.d.	n.d.	26.02	n.d.	98.06	n.d.	n.d.	n.d.	335.47	n.d.	n.d.	n.d.
1-octen-3-ol	n.d.	n.d.	1.02	n.d.	7.32	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fenchyl alcohol	n.d.	n.d.		59.79	n.d.	n.d.	n.d.	n.d.	n.d.	21.34	n.d.	n.d.
2-ethylhexanol	0.86	1.84	2.38	14.94	3.03	9.51	1.50	n.d.	3.83	8.62	n.d.	n.d.
4-hepten-1-ol	n.d.	n.d.	n.d.	n.d.	n.d.	8.72	n.d.	n.d.	n.d.	3.72	n.d.	n.d.
2,3-butanediol	n.d.	2.11	n.d.	84.79	6.18	81.54	n.d.	54.10	n.d.	82.21	n.d.	48.24
1-octanol	1.37	2.73	n.d.	n.d.	10.99	54.94	n.d.	n.d.	32.56	98.05	n.d.	n.d.
2,3-butanediol (isomero)	n.d.	1.62	n.d.	12.31	n.d.	60.56	n.d.	36.31	n.d.	12.28	n.d.	46.23
Terpinen-4-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.81	4.82	n.d.	n.d.	n.d.	n.d.
cis-6-nonenol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	26.74	182.45	n.d.	n.d.
Benzyl alcohol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.99	n.d.	2.52
Phenylethylalcohol	n.d.	117.29	n.d.	588.26	0.69	2241.74	n.d.	1002.72	n.d.	514.19	n.d.	1438.45
Glycerol	n.d.	2.03	n.d.	297.53	n.d.	89.40	n.d.	98.79	n.d.	3.56	n.d.	104.63
Total	77.21	639.02	410.07	4924.05	252.74	6727.80	73.04	1369.16	1107.72	3551.64	414.57	4449.04
Aldehydes												
Hexanal	74.24	n.d.	183.01	n.d.	532.04	16.17	3.35	n.d.	74.46	46.08	669.77	n.d.
2-hexenal	n.d.	n.d.	78.90	n.d.	178.34	n.d.	n.d.	n.d.	60.15	n.d.	62.26	n.d.
1-octanal	n.d.	n.d.	n.d.	n.d.	43.97	n.d.	2.29	n.d.	n.d.	98.19	n.d.	n.d.
Nonanal	6.53	4.74	6.90	21.70	40.35	19.67	n.d.	n.d.	35.49	28.85	7.53	9.67
trans-2-octenal	n.d.	n.d.	n.d.	n.d.	94.59	35.51	0.87	n.d.	106.99	86.41	n.d.	n.d.
Decanal	3.04	2.91	3.57	6.32	6.85	12.03	0.79	9.66	n.d.	8.10	11.18	2.95
4-methylbenzaldehyde	9.23	8.07	n.d.	n.d.	14.98	233.76	45.04	131.33	n.d.	n.d.	n.d.	n.d.
Benzaldehyde	3.26	3.03	3.03	16.95	4.23	12.66	1.57	n.d.	4.59	29.69	11.69	40.69
β-cyclocitral	n.d.	n.d.	n.d.	n.d.	4.41	6.14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenylethanal	n.d.	n.d.	33.06	n.d.	n.d.	n.d.	n.d.	n.d.	28.88	n.d.	27.79	286.93
Geranial	n.d.	n.d.	1.16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	87.08	10.69	309.61	44.98	904.79	102.18	8.87	9.66	310.56	297.32	790.22	340.24
Diverse functional groups												
Hydroxyacetone	0.77	5.46	0.97	36.22	n.d.	52.27	1.35	89.68	2.95	7.71	n.d.	n.d.
trans-linaloloxide	n.d.	n.d.	5.33	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Furfuraldehyde	1.04	3.97	1.88	n.d.	n.d.	70.97	2.31	59.30	n.d.	24.41	47.96	99.94
5-methylfurfural	0.65	0.71	1.91	453.57	n.d.	n.d.	1.65	28.59	n.d.	n.d.	9.56	27.89
2-penthylfuran	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.68	n.d.	18.65	n.d.	n.d.	n.d.
Furfuryl alcohol	0.32	2.47	n.d.	846.02	n.d.	18.48	0.57	50.72	n.d.	n.d.	30.44	44.80
5-hydrxoymethylfurfural	2.75	10.04	2.98	55.72	7.47	121.83	3.42	182.42	8.39	129.94	77.85	175.89
Total	5.54	22.65	13.08	1391.53	7.47	263.56	9.30	410.70	11.34	162.06	165.81	348.51
Esters												
Isoamylacetate	7.32	331.27	n.d.	4136.48	n.d.	1618.69	n.d.	2803.77	n.d.	86.21	n.d.	3047.23
Methylhexanoate	n.d.	n.d.	n.d.	n.d.	14.57	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethyl hexanoate	n.d.	515.01	n.d.	8424.13	9.68	4521.45	n.d.	6193.08	n.d.	663.74	n.d.	5987.32
Hexyl acetate	6.87	737.67	n.d.	3279.88	1.53	3404.19	3.91	172.10	10.72	400.71	2.11	1833.43
cis-3-hexenyl acetate	n.d.	1.31	n.d.	15.77	n.d.	6.45	n.d.	59.71	n.d.	n.d.	36.10	170.08
Ethyl heptanoate	n.d.	9.10	n.d.	359.47	n.d.	29.30	n.d.	n.d.	n.d.	n.d.	n.d.	71.00
Isobutyl hexanoate	n.d.	1.13	n.d.	36.14	n.d.	7.86	n.d.	5.40	n.d.	n.d.	n.d.	8.50
Ethyl lactate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.96	n.d.	n.d.
1-heptyl acetate	n.d.	6.33	n.d.	807.31	n.d.	3827.96	n.d.	n.d.	n.d.	2.93	n.d.	45.00
Methyloctanoate	n.d.	0.92	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethyl octanoate	1.59	2247.26	2.21	58169.56	30.69	1833.20	8.44	8446.21	9.23	3444.50	5.45	5337.65

Isoamyl hexanoate	n.d.	15.87	n.d.	486.31	n.d.	45.21	n.d.	n.d.	n.d.	11.98	n.d.	16.74
Octyl acetate	n.d.	4.93	n.d.	102.09	n.d.	2.64	n.d.	13.75	n.d.	19.49	n.d.	5.39
Ethyl nonanoate	n.d.	1.72	n.d.	106.83	n.d.	59.03	n.d.	5.05	n.d.	168.11	n.d.	12.72
Isobutyloctanoate	n.d.	2.74	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isoamyl lactate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.10	n.d.	n.d.
Methyl decanoate	n.d.	0.74	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hexyl hexanoate	0.33	6.22	n.d.	23.72	n.d.	n.d.	n.d.	n.d.	n.d.	20.09	n.d.	n.d.
γ-butyrolactone	n.d.	n.d.	1.08	n.d.	n.d.	n.d.	n.d.	12.29	n.d.	n.d.	4.74	5.54
Ethyldecanoate	0.50	592.64	n.d.	43.27	12.45	308.47	3.52	1962.47	2.74	909.10	n.d.	1041.10
Isoamyl octanoate	n.d.	24.83	n.d.	22069.03	n.d.	8.32	n.d.	25.26	n.d.	32.78	n.d.	34.84
Estragole	13.44	9.79	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethyl-9-decenoate	n.d.	208.98	n.d.	14.38	n.d.	76.85	n.d.	122.73	n.d.	181.02	n.d.	367.74
2(5H)-furanone	1.26	1.71	1.59	n.d.	1.93	11.32	1.17	24.10	2.40	8.37	7.86	20.79
Methyl salicylate	n.d.	n.d.	n.d.	n.d.	n.d.	1.92	3.31	17.89	n.d.	33.14	n.d.	n.d.
Phenylethylacetate	n.d.	75.07	n.d.	6343.85	n.d.	432.92	n.d.	620.66	n.d.	68.64	n.d.	690.18
Ethyldodecanoate	n.d.	181.12	n.d.	893.00	6.64	64.54	2.54	431.06	n.d.	145.92	n.d.	298.73
Isoamyl decanoate	n.d.	5.65	n.d.	162.29	n.d.	2.89	n.d.	10.88	n.d.	6.16	n.d.	10.75
Ethyl tetradecanoate	n.d.	8.22	n.d.	100.83	n.d.	2.57	0.79	17.70	n.d.	10.13	n.d.	14.70
Isoamyl dodecanoate	n.d.	0.76	n.d.	4.85	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-phenylethyl hexanoate	n.d.	n.d.	n.d.	34.59	n.d.	13.23	n.d.	9.74	n.d.	1.94	n.d.	14.64
Myristicin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.25	n.d.	n.d.
Ethyl hexadecanoate	n.d.	9.62	n.d.	26.51	n.d.	n.d.	0.77	10.98	5.30	21.48	n.d.	13.15
Phenylethyl octanoate	n.d.	9.88	n.d.	43.20	n.d.	4.52	n.d.	11.81	n.d.	1.49	n.d.	15.88
Coumaran	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	17.49	n.d.	n.d.	n.d.	n.d.
Total	45.13	5034.14	10.59	106371.09	102.81	16343.03	38.45	21071.39	49.05	6858.02	62.24	19128.03
Ketones												
Acetoin	n.d.	1.91	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.13	9.97
6-methyl-5-heptene-2-one	0.43	n.d.	1.73	3.99	16.73	26.96	0.95	2.93	n.d.	20.57	3.53	n.d.
α-ionone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	27.36	n.d.	n.d.	n.d.
Geranylacetone	n.d.	n.d.	n.d.	n.d.	8.64	n.d.	n.d.	n.d.	n.d.	18.91	n.d.	n.d.
β-ionone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	23.84
1-(3-erthylphenyl)ethanone	1.13	1.94	1.44	31.61	2.41	8.64	1.70	16.48	n.d.	9.38	3.67	16.36
Total	1.57	1.94	3.16	35.61	27.77	35.60	2.65	19.41	27.36	48.86	7.20	40.20
Phenols												
Phenol	0.93	1.71	n.d.	n.d.	n.d.	n.d.	1.07	11.60	n.d.	n.d.	n.d.	n.d.
Eugenol	n.d.	13.89	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tymol	n.d.	n.d.	n.d.	n.d.	n.d.	3.16	n.d.	13.64	n.d.	11.85	n.d.	n.d.
Total	0.93	15.60	0.00	0.00	0.00	3.16	1.07	25.23	0.00	11.85	0.00	0.00
Sulphur compounds												
3-(methylthio)propanol	n.d.	1.05	n.d.	n.d.	n.d.	39.42	n.d.	17.89	n.d.	44.35	n.d.	7.90
Aromatic Hydrocarbons												
Styrene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	47.20	n.d.	79.82	0.74	12.00
p-cymene	n.d.	n.d.	1.33	85.24	n.d.	n.d.	1.26	n.d.	n.d.	46.68	n.d.	n.d.
2.5-dimethylstyrene	n.d.	n.d.	0.91	n.d.	n.d.	n.d.	0.62	5.36	n.d.	n.d.	n.d.	n.d.
Total	0.00	0.00	2.24	85.24	0.00	0.00	1.88	52.56	0.00	126.51	0.74	12.00
Terpens and terpenols												
β-pinene	n.d.	n.d.		n.d.	n.d.	n.d.	1.27	n.d.	n.d.	n.d.	n.d.	n.d.
β-myrcene	n.d.	n.d.	23.87	n.d.	n.d.	n.d.	n.d.	n.d.	4.46	n.d.	n.d.	n.d.
D-limonene	n.d.	n.d.	11.11	376.80	n.d.	14.61	6.82	n.d.	n.d.	65.14	n.d.	n.d.
β-phellandrene	n.d.	n.d.	1.81	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	65.14	n.d.	n.d.
3-carene	n.d.	n.d.	8.46	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Linalol	n.d.	n.d.	262.41	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
α-terpinolene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.76	n.d.	n.d.	n.d.	n.d.	n.d.
β-caryophyllene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.68	n.d.	n.d.	n.d.	n.d.	n.d.
Anethol	13.82	23.65	5.71	687.62	25.33	59.52	13.33	77.26	n.d.	605.80	5.99	64.96
β-farnesene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	49.44	n.d.	n.d.
α-tepineol	n.d.	n.d.	3.03	n.d.	n.d.	n.d.	6.90	12.03	n.d.	n.d.	n.d.	n.d.
δ-guaiene	0.47	0.86	n.d.	n.d.	n.d.	25.77	6.44	5.61	n.d.	n.d.	5.86	27.11
Citronellol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	72.15	7.69	n.d.	n.d.
Geraniol	n.d.	n.d.	9.50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Curcumene	n.d.	n.d.	n.d.	n.d.	n.d.	3.05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	9.70	8.93	45.26	376.80	14.98	277.19	63.02	136.94	4.46	179.72	5.86	27.11

Results indicate mean values of three measurements and are expressed (in µg/L) as 1-Heptanol.
The chemicals are shown following their retention time.
Abbreviations: FJ, fruit juice; KLB, kefir-like beverage; n.d. not detected.

Legend to figures

Fig. 1. Representative polymorphic profiles of LAB and yeast colonies isolated from the commercial freeze-dried water kefir starter culture and KLBs. Lanes: M, marker; 1, *Leuconostoc mesenteroides* (Acc. No. KT633927); 2, *Lactococcus lactis* (Acc. No. KT633921); 3, *Lactobacillus kefiri* (Acc. No. KT633919); 4, *Lactobacillus fermentum* (Acc. No. KT633923); 5, *Saccharomyces cerevisiae* (Bankit 1853683).

Fig. 2. Overall quality of Mediterranean fruit-based kefir-like beverages. Bars with the same letter are not statistically different at $P<0.05$ (Tukey–Kramer's multiple range test).

Fig. 3. PCA analysis based on the values of microbial loads on kefir-like beverages, biplot graphs shows relationships among factors, variables and samples. Abbreviations: PCA, plate count agar for total mesophilic counts; VRBGA, violet red bile glucose agar for *Enterobacteriaceae*; PAB, *Pseudomonas* agar base for pseudomonads; MRS, de Man-Rogosa-Sharpe agar for rod LAB; M17, medium 17 agar for mesophilic coccus LAB; DRBC, dichloran rose Bengal chloramphenicol agar for yeasts; KLB, kefir-like beverage; Δ values are referred to the differences on the microbial loads between the fruit juices and the corresponding kefir-like beverage for each medium.

Fig. 4. PCA analysis based on the values of physico-chemical determinations on kefir-like beverages, biplot graphs shows relationships among factors, variables and samples. Abbreviations: CO_2, carbon dioxide; TTA, total titratable acidity; SSC, soluble solid content; PT, total phenol (gallic acid equivalent mg/L); DPPH, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (%); ΔE, color differences.

Fig. 5. PCA analysis based on the values of volatile organic compounds on kefir-like beverages, biplot graphs shows relationships among factors, variables and samples.

Fig. 1.

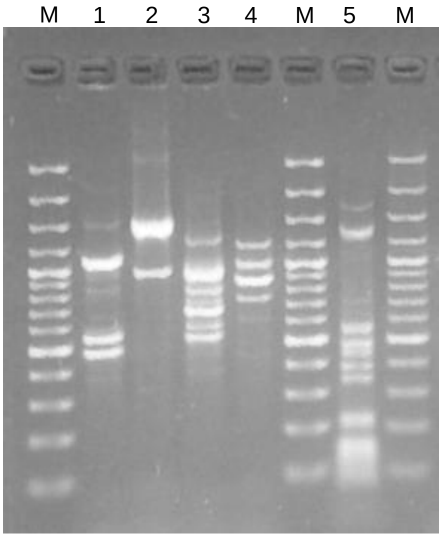
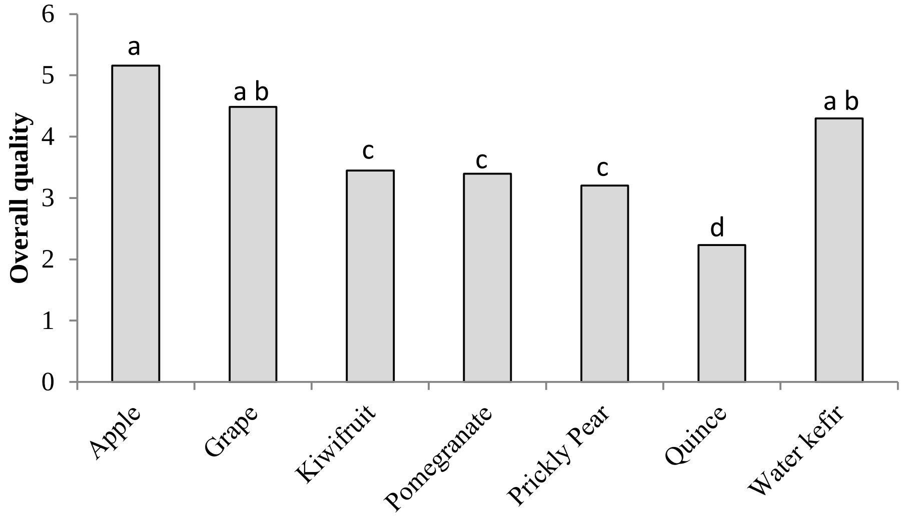
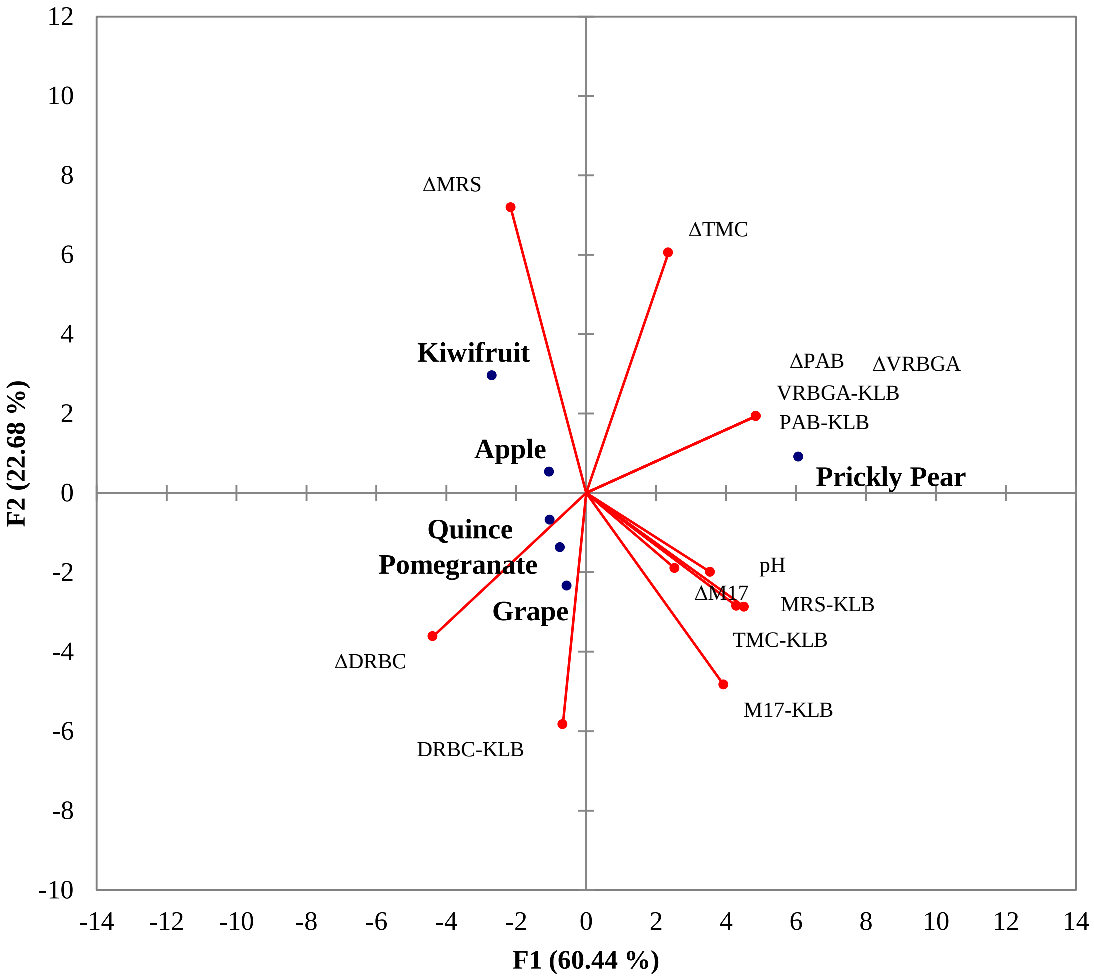


Fig. 2.



Overall quality
0
1
2
3
4
5
6
a
a b
c
c
c
d
a b
Apple
Grape
Kiwifruit
Pomegranate
Prickly Pear
Quince
Water kefir

679 **Fig. 3.**



680

681

682 **Fig. 4.**

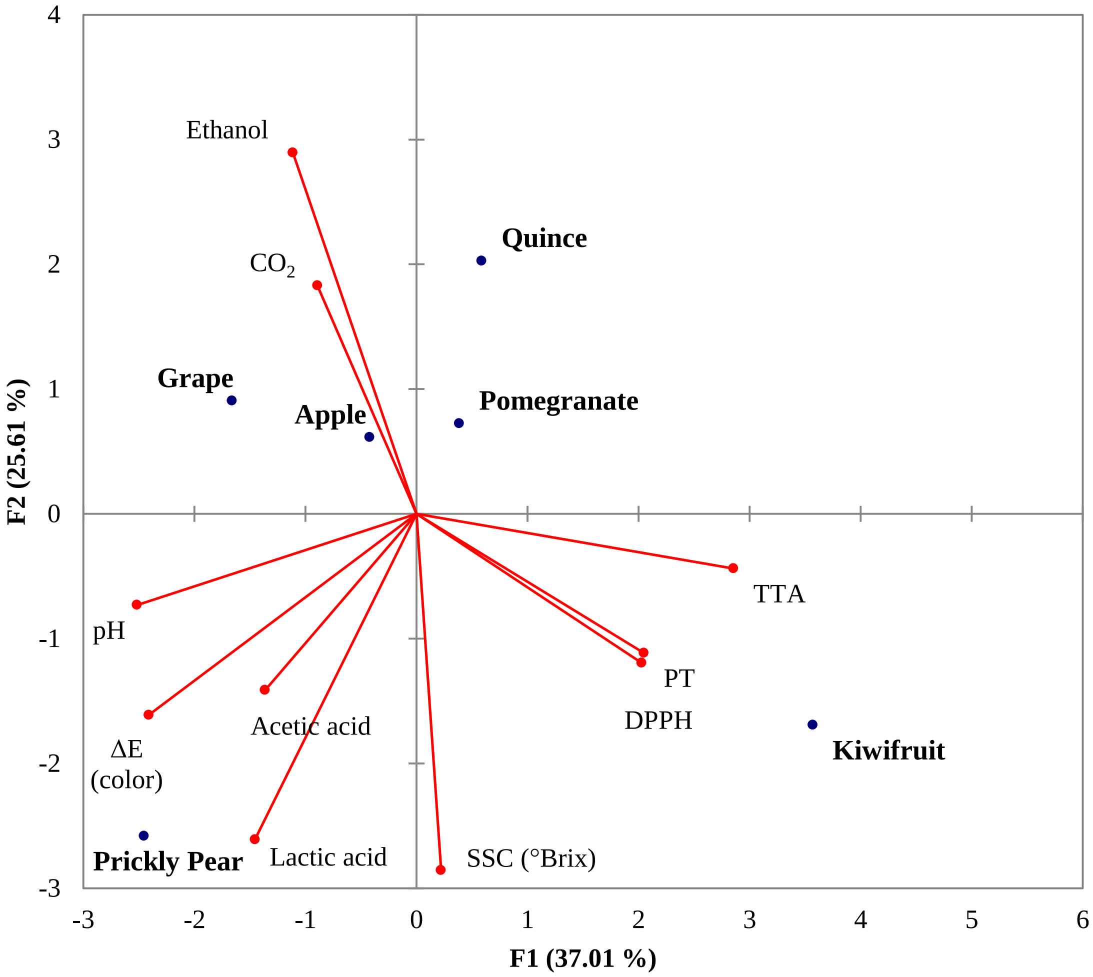


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Fig. 5.

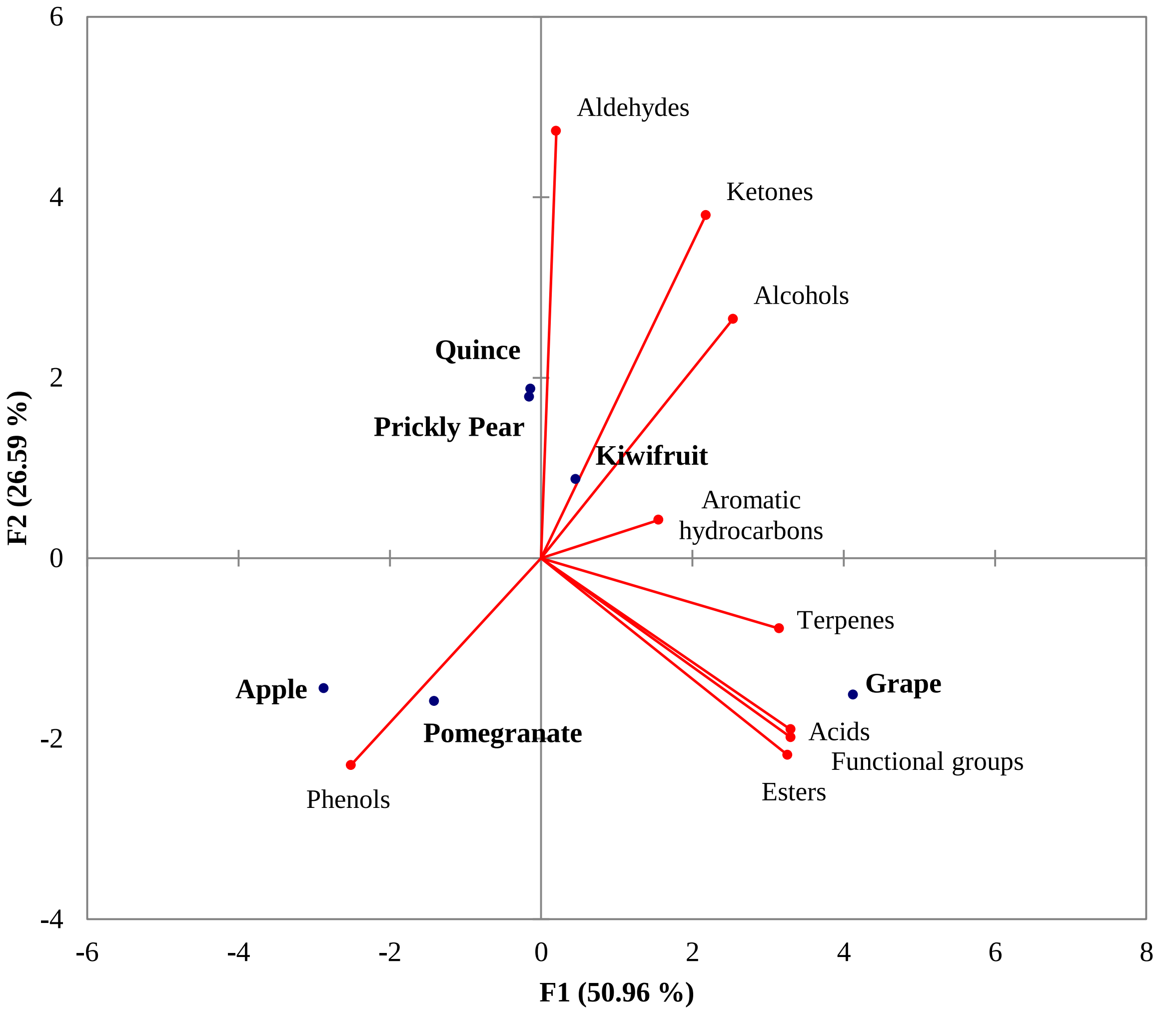
Aldehydes
Ketones
Alcohols
Quince
Prickly Pear
Kiwifruit
Aromatic hydrocarbons
Terpenes
Grape
Apple
Pomegranate
Acids
Functional groups
Esters
Phenols
F2 (26.59 %)
F1 (50.96 %)
6
4
2
0
-2
-4
-6
-4
-2
0
2
4
6
8

Table S1 Supplemental material Correlation of microbial variables to the factors of the PCA based on factor loadings**.**

	F1	F2	F3	F4	F5
TMC - KLB	**0.902**	-0.351	0.245	-0.058	0.025
VRBGA - KLB	**0.969**	0.236	0.056	-0.045	-0.024
PAB - KLB	**0.969**	0.236	0.056	-0.045	-0.024
MRS - KLB	**0.857**	-0.348	-0.113	-0.247	-0.267
M17 - KLB	**0.784**	-0.590	-0.089	0.042	-0.165
DRBC - KLB	-0.134	**-0.712**	**0.616**	-0.254	0.174
ΔTMC	0.469	**0.739**	0.385	-0.239	0.168
ΔVRBGA	**0.969**	0.236	0.056	-0.045	-0.024
ΔPAB	**0.969**	0.236	0.056	-0.045	-0.024
ΔMRS	-0.430	**0.878**	0.123	0.102	-0.135
ΔM17	0.505	-0.232	0.402	**0.728**	0.082
ΔDRBC	**-0.874**	-0.442	0.132	-0.122	-0.093
pH	**0.707**	-0.243	-0.571	0.072	0.332

Abbreviations: TMC, total microbial count; VRBGA, violet red bile glucose agar for *Enterobacteriaceae*; PAB, *Pseudomonas* agar base for pseudomonads; MRS, Man-Rogosa-Sharpe agar for mesophilic rod LAB; M17, for lactic streptococci; DRBC, dichloran rose bengal chloramphenico agar for total yeasts and filamentous fungi; pH, values of pH of KLB; Δ, increase of microbiological count of KLB respect to the unfermented products.
Values in bold within the same factor indicate the variable with the largest correlation.

Table S2 Supplemental material Correlation of physico-chemical variables to the factors of the PCA based on factor loadings**.**

	F1	F2	F3	F4	F5
pH	**-0.822**	-0.198	0.243	-0.449	-0.153
TTA	**0.933**	-0.119	-0.144	0.308	-0.001
SSC	0.072	**-0.776**	0.215	-0.406	0.426
TP	**0.669**	-0.303	**0.667**	0.052	0.115
DPPH	**0.663**	-0.325	**0.634**	0.146	-0.179
ΔE	**-0.787**	-0.438	0.365	0.204	0.117
Ethanol	-0.364	**0.787**	0.393	0.227	0.207
Lactic acid	-0.475	**-0.709**	0.118	0.342	-0.374
Acetic acid	-0.446	-0.384	-0.381	**0.659**	0.273
CO_2	-0.292	0.497	**0.791**	0.204	0.008

Abbreviations: pH, pH of KLB; TTA, Total Titatrable Acidity; SSC, Soluble solid content; TP, Total phenolic compounds; DPPH, radical scavenging activity; ΔE, color difference parameter, CO_2, carbon dioxide.
Values in bold within the same factor indicate the variable with the largest correlation.

Table S3 Supplemental material Correlation of volatile organic compounds variables to the factors of the PCA based on factor loadings**.**

	F1	F2	F3	F4	F5
Acids	**0.896**	-0.372	-0.038	-0.238	0.035
Alcohol	**0.689**	0.520	-0.375	0.334	0.050
Aldehydes	0.054	**0.928**	0.160	-0.329	0.038
Functional groups	**0.896**	-0.389	0.032	-0.180	0.113
Esters	**0.884**	-0.428	-0.013	-0.187	-0.001
Ketons	0.591	**0.745**	0.272	0.078	0.121
Phenols	**-0.682**	-0.450	0.500	0.203	0.203
Aromatic hydrocarbons	0.421	0.083	**0.893**	0.010	-0.134
Terpens	**0.855**	-0.153	0.083	0.487	-0.052

Values in bold within the same factor indicate the variable with the largest correlation.