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# Study of kefir drinks produced by backslopping method using kefir grains from Bosnia and Herzegovina: microbial dynamics and volatilome profile

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**Abstract**

Kefir is a well-known health-promoting beverage that can be produced by using kefir grains (traditional method) or by using natural starter cultures from kefir (backslopping method). The aim of this study was to elucidate the microbial dynamics and volatilome profile occurring during kefir production through traditional and backslopping methods by using five kefir grains that were collected in Bosnia and Herzegovina. The results from conventional pour plating techniques and amplicon-based sequencing were combined. The kefir drinks have also been characterized in terms of their physico-chemical and colorimetric parameters. A bacterial shift from *Lactobacillus kefiranofaciens* to *Acetobacter syzygii*, *Lactococcus lactis* and *Leuconostoc pseudomesenteroides* from kefir grains in traditional kefir to backslopped kefir was generally observed. Despite some differences within samples, the dominant mycobiota of backslopped kefir samples remained quite similar to that of the kefir grain samples. However, unlike the lactic acid and acetic acid bacteria, the yeast counts decreased progressively in the grains from the backslopped kefir. The backslopped kefir samples showed higher protein, lactose and ash content and lower ethanol content compared to traditional kefir samples, coupled with optimal pH values that contribute to a pleasant sensory profile. Concerning the volatilome, backslopped kefir samples were correlated with cheesy, buttery, floral and fermented odors, whereas the traditional kefir samples were correlated with alcoholic, fruity, fatty and acid odors. Overall, the data obtained in the present study provided evidence that different kefir production methods (traditional vs backslopping) affect the quality characteristics of the final product. Hence, the functional traits of backslopped kefir should be further investigated in order to verify the suitability of a potential scale-up methodology for backslopping.

**Keywords:** kefir grain microbiota, backslopped kefir, physico-chemical parameters, viable counts, Illumina sequencing, VOCs, SPME-GC/MS

## 1. Introduction

Kefir is a self-carbonated, acidic and low-alcohol fermented milk beverage characterized by a unique volatile profile and nutritional composition and a creamy texture (Gao & Li, 2016; Prado et al., 2015). Kefir originated from Caucasian and Balkans populations, and over time, its popularity and consumption have increased around the world due to its sensory properties, health-promoting features and its status of natural probiotic. All these features are related to the kefir microorganisms, their interactions and to their metabolic products such as lactic and acetic acids, carbon dioxide, ethanol, acetaldehyde, acetoin, and other volatile compounds, minerals, essential amino acids, vitamins (B1, B2, B5, B12, K and C), folic acid, bacteriocins, bioactive peptides, some nutraceutical components and exopolysaccharides (EPS) as kefiran (Arslan, 2015; Bourrie, Willing, & Cotter, 2016; Farnworth, 2005; Guzel-Seydim, Kök-Taş, Greene, & Seydim, 2011; Leite et al., 2013a; Prado et al., 2015). Traditionally, kefir is obtained through the fermentation of milk by kefir grains, which are irregular, lobed-shaped, gelatinous and slimy structures with variable dimensions (from 1 to 4 cm in length) and colors (from white to light yellow) (Garofalo et al., 2015; Leite et al., 2013a; Prado et al., 2015). Kefir grains are made up of a natural matrix of EPS (kefiran) and proteins in which lactic acid bacteria (LAB), yeasts and sometimes acetic acid bacteria (AAB) coexist in symbiotic association (Garofalo et al., 2015; Leite et al., 2013a; Prado et al., 2015). At the artisanal/homemade level, kefir grains represent the starter culture for kefir production. Specifically, the grains are added to milk at variable ratios (generally from 1 to 20% w/v) and are left to ferment for 18-24 h at 20-25°C (Leite et al., 2013a). During fermentation, the grains increase in biomass, and they may break into new, smaller grains and release viable cells into the substrate (Marshall & Cole, 1985; Prado et al., 2015). At the end of fermentation, the grains are separated from kefir by sieving, and they can be used for the next fermentation (Leite et al., 2013a).

At the commercial level, kefir can be manufactured by i) the fermentation of milk with pure freeze-dried commercial kefir cultures or ii) by using the so-called “Russian method”, in which the kefir is

produced at a larger scale than the traditional method through backslopping, a process of fermentation in series that starts from the kefir produced with grains that is then used as natural starter cultures for milk fermentation (Leite et al., 2013a; Prado et al., 2015).

Although producing kefir with commercial starter cultures leads to a more standardized product compared with kefir produced using kefir grains, health benefits have been found to be linked only to kefir produced with the complex microbiota derived from grains or grain-fermented milks (Bourrie et al., 2016; Leite et al., 2013a). In fact, the microbiota of kefir grains may vary depending on the ratio of kefir grains to milk, the time and temperature of fermentation (which are strictly connected to the geographical origin of kefir grains and thus to the climate conditions) and the type of substrate used for fermentation, which is generally bovine milk, although buffalo, camel, sheep, goat, rice, coconut and soy drinks have also been used (Altay, Karbancıoglu-Güler, Daskaya-Dikmen, & Heperkan, 2013; Bourrie et al., 2016). As a consequence of the different microbial consortium that can become established within kefir grains, variable kefir products with different microbiological, physico-chemical, nutritional and sensorial properties may be obtained (Bengoa, Iraporda, Garrote, & Abraham, 2018). This last aspect justifies the huge number of scientific studies on microbiological aspects of kefir grains collected from several countries around the world (Prado et al., 2015). Surprisingly, although kefir originated in the Balkans, to the authors' knowledge, few scientific studies have been published on microbiota characterizations of kefir grains from this area (Bulgaria; Simova et al., 2002), (Turkey; Guzel-Seydim, Wyffels, Seydim, & Greene, 2005; Kesmen & Kacmaz, 2011; Kök-Taş, Ekinci, & Guzel-Seydim, 2012), with none from Bosnia and Herzegovina. Furthermore, relatively few studies have been published to compare the microbiota between kefir grains and the related kefir drinks obtained using the traditional method (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011; Gao & Zhang, 2019; Guzel-Seydim et al., 2005; Kesmen & Kacmaz, 2011; Korsak et al., 2015; Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013), and to the author's knowledge, there are none published data on microbial dynamics and volatilome profile that occur during the production of kefir through backslopping. To date, the study by Marshall & Cole (1985) first

described the backslopping method for kefir production, and later, Simova et al. (2002) investigated the changes in the LAB and yeast communities during the kefir backslopping production process by using a culture-dependent technique and by identifying isolates with phenotypic methods. Satir & Guzel-Seydim later (2016) applied the same fermentation methods to milks from different caprine and bovine species and analyzed the products from a chemical and nutritional point of view. Recently, Kim, Jeong, Song, & Seo (2018) characterized the microbial populations of traditional kefir and backslopped kefir by using a culture-dependent technique and species-specific quantitative real-time PCR to quantify the *Lactobacillus kefiranofaciens*, *Lactobacillus kefiri* and *Kluyveromyces marxianus* (considered as key functional microorganisms in kefir), coupled with physico-chemical, nutritional and sensorial characterizations of these kefir drinks.

Therefore, the aims of this study were i) the elucidation of the bacterial and fungal community compositions in five kefir grains collected in Bosnia and Herzegovina; ii) a study of the microbial dynamics that occur during the production of kefir by traditional and backslopping methods, by performing viable counts on selective culture media and amplicon-based sequencing; and iii) a full characterization of the related kefir drinks in terms of their physico-chemical parameters, colorimetric features and volatilome profiles.

## 2. Material and methods

### *2.1. Origin, maintenance of milk kefir grains and kefir production*

Five milk kefir grains (KGA, KGB, KGC, KGD, and KGE) were obtained from private households located in Bosnia and Herzegovina. These kefir grains were activated in the laboratory as described below. The kefir grains were inoculated (10% w/v) into sterile whole bovine (U.H.T.) milk and incubated at 22 °C for 24 h. After this time, the grains were filtered through a sterilized plastic sieve, they were gently washed with sterile distilled water, and subsequently, they were inoculated into milk

again for a daily fermentation at 22 °C. This procedure was repeated for 1 month to keep the grains active and to increase the grain biomass.

Five kefir drinks were produced by traditional method (by using each type of kefir grains) or by backslopping method (by using the fermented milk obtained from each kefir grain fermentation) as follows. Twenty grams of each grain were inoculated (10% w/v) into 200 mL of sterile whole bovine (U.H.T.) milk (12.14% dry matter, 3.74% fat, 3.35% protein, 4.65% lactose, and 0.74% ash) and incubated at 22 °C for 24 h.

After the incubation, the grains were separated by filtration from the fermented milks. The filtrate that corresponded to the traditional kefir (hereafter referred to as TK) was also used as natural starter culture to produce kefir by backslopping (hereafter referred to as BK). Specifically, 50 mL aliquots of each TK were used to inoculate 1 L of sterile U.H.T. whole bovine milk (5% v/v). The fermentation was performed statically at 22 °C for 24 h (Puerari, Teixeira Magalhães, & Freitas Schwan, 2012; Satir & Guzel-Seydim, 2016). The preparation procedure for TK and BK is reported in Supplementary Figure 1.

The kefir production was performed in duplicates (Puerari et al., 2012) and considered in blocks during the data analysis.

*2.2. Enumeration of culturable bacteria and yeasts in milk kefir grains and drinks*

Ten grams of each kefir grain (KGA-KGE) was homogenized in 90 mL of cold sterile 0.1% peptone solution using a Stomacher apparatus (400 Circulator, International PBI) for 15 min at maximum speed (260 rpm) (Garofalo et al., 2015).

Ten milliliters of each kefir drink, namely traditional kefir (TKA-TKE) and backslopped kefir (BKA-BKE), was diluted in 90 mL of cold sterile 0.1% peptone solution.

All of the samples were serially diluted. Serial decimal dilutions were prepared in cold sterile 0.1% peptone solution, and 0.1 mL of each serial dilution was inoculated by spreading it onto the surface of specific solid media in duplicate. The following microorganisms were counted: (i) LAB on MRS

agar (Difco, Sparks, MD, USA) at 37 °C under anaerobic conditions (Aquilanti et al., 2012); (ii) AAB on Gluconobacter Medium (GM) agar (mannitol 2.5%, yeast extract 0.5%, peptone 0.3%) and these media were supplemented with 400 mg/L of cycloheximide to inhibit yeast growth; and (iii) yeasts on Rose Bengal Chloramphenicol Agar (RBCA) (Difco) under aerobic conditions at 25 °C. The microbial enumerations of the bacteria and yeasts were performed after 3 and 10 days, respectively. The results of the viable counts were expressed as the means of the log of the colony forming units (CFU) per gram or mL of kefir grains or kefir drinks, respectively.

*2.3. DNA extraction from milk kefir grains and drinks*

The microbial DNA was extracted directly from the milk kefir grains (KGA-KGE) and kefir drinks (TKA-TKE and BKA-BKE) using a PowerFood™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA). Specifically, 1 mL of each kefir grain homogenate (dilution $10^{-1}$) used for microbial plating was centrifuged at 13.000 g for 3 min to produce a pellet, while 1.5 mL of each kefir drink was centrifuged at 13.000 g for 3 min. Each pellet (from the kefir grains and kefir drinks) was processed according to the kit manufacturer's instructions.

*2.4 Analysis of bacterial and yeast diversity by rRNA gene Illumina sequencing and bioinformatics and data analysis*

*2.4.1 Amplicon target sequencing*

A total of 30 DNA extracts were obtained from the two kefir productions, each including 5 kefir grains, 5 TK samples and 5 BK samples. The DNA extracts were quantified using a QUBIT dsDNA assay kit (Life Technologies, Milan, Italy) and they were standardized to 20 ng/µL. The DNA extracts from the two kefir productions were then pooled and used to amplify the V3-V4 region of the 16S rRNA gene (Klindworth et al., 2013) as well as the D1 domain of the 26S rRNA gene (Mota-Gutierrez et al., 2018).

PCR was performed for the two target regions using the following PCR mixture: 12.5 μL of the 2× Kapa HiFi HotStart ReadyMix Taq (Roche, Milan, Italy), 5 μL of each primer (1 μM), and 2.5 μL of DNA as a template. The PCR mixtures were subjected to the following amplification conditions: thirty cycles with 30 s of denaturation (95 °C), 30 s of primer annealing (55 °C), and 30 s of primer elongation (72 °C), followed by a final elongation step (72 °C) of 10 min.

The PCR amplicons were purified and sequenced according to the Illumina metagenomic pipeline instructions. The sequencing was performed with a MiSeq Illumina instrument (Illumina) with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instructions.

*2.4.2 Bioinformatics analysis*

After the sequencing, the raw reads were merged with FLASH software (Magoc & Salzberg, 2011) and the resulting reads were analyzed with QIIME 1.9.0 software (Caporaso et al., 2010); the relevant pipeline was recently described (Ferrocino et al., 2017). For the 16S data, the centroid sequences of each OTU cluster was mapped against Greengenes 16S rRNA gene database version 2013 for taxonomic assignment, while for the 26S data, the in-house database from Mota-Gutierrez et al. (2018) was used.

To avoid biases due to the different sequencing depths, each dataset was rarefied at the lowest number of reads; the 26S data were rarefied at 37376 sequences while 7969 sequences were chosen for 16S data. The OTU tables generated through QIIME showed the highest taxonomy resolution that was reached. The sequences were double-checked using the BlastN search tool (http://www.ncbi.nlm.nih.gov/blast/) to confirm the taxonomy assignment.

The 16S and 26S rRNA gene sequences are available at the Sequence Read Archive of the NCBI (accession number SRP217033).

*2.5 Physico-chemical measurement of kefir drinks*

The pH of the kefir drinks (TKA-TKE and BKA-BKE) was measured at room temperature using a model 300 pH meter equipped with an HI2031 solid electrode (HI2031, Hanna Instruments, Padua, Italy).

For the total titratable acidity (TTA) determination, 10 mL of kefir drinks was mixed with 90 mL of distilled water, stirred with a magnetic stirrer and then titrated with 0.1 N NaOH. The results were expressed as the mean amounts of the NaOH (mL) used to reach a pH of 8.3. All the assays were performed in triplicate.

*2.6 Proximate composition of kefir drinks*

The kefir drinks (TKA-TKE and BKA-BKE) and milk were analyzed for their moisture, dry matter, fat, protein, lactose and ash contents. These analyses were all performed in the same accredited laboratory (ACCREDIA, accreditation No. 0217). The fat %, protein %, and lactose %, as expressed in w/w, were quantified via Fourier Transform Infrared (FTIR) spectroscopy using a CombiFoss FT+ made up of a Milkoscan FT Plus – 300 and a Fossomatic FC (Foss Electric, Hillerød, Denmark); the dry matter and ash were analyzed according to AOAC methodologies (950.46; 920.153). All these chemical analyses were performed in duplicate and the results were expressed as a % (w/w).

*2.7 D-/L-lactic acid determination*

The concentration of D-/L-lactic acid in the kefir drinks (TKA-TKE and BKA-BKE) was quantified using a Megazyme Assay Kit (K-DLATE 11/17) (MEGAZYME International, Wicklow, Ireland, 2017) according to the manufacturer's instructions. All the samples were examined in triplicate.

2.*8 Color properties of kefir drinks*

The colorimetric profile of the kefir drinks (TKA-TKE and BKA-BKE) was measured with a Chroma Meter CR-200 (Minolta, Japan) to determine the L (lightness) and chromaticity coordinates (a* and b* expressing redness and yellowness, respectively) according to CIELab. The color intensity was

determined by calculating the chroma index according to the following equation: $\sqrt{(a^{*2} + b^{*2})}$. The set-up for the Chroma Meter was performed on a white reference standard plate with a D65 illuminant characterized by a visible spectrum similar to that of natural light. The colorimetric readings were performed in triplicate for each sample.

*2.9 Volatile organic compounds (VOCs) of kefir drinks*

*2.9.1 Characterization of volatile organic compounds (VOCs) in kefir drinks*

The volatile fraction of the kefir drinks (TKA-TKE and BKA-BKE) was analyzed by headspace sampling, using Solid Phase Micro-Extraction coupled with Gas Chromatography/Mass Spectrometry (SPME-GC/MS). For each SPME analysis, 5 g of sample was placed inside a 20 mL headspace vial, which was immediately sealed with a Teflon-lined septum and screwcap. The vial was placed in a thermostatic block on a stirrer at 55 °C for 5 min. After equilibration, the headspace of the samples was sampled using an SPME fiber coated with DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane, thickness 50/30 mm) at 55 °C for 50 min. The HS-SPME was automatically performed with a multipurpose sampler (Gerstel MPS2) (Dertli & Çon, 2017).

*2.9.2 Gas chromatography/mass spectrometry (GC/MS) analysis*

The volatile analysis was performed using an Agilent Technologies 7890A GC coupled to an Agilent Technologies 5975 mass spectrometer (Santa Clara, California) equipped with a 30 m x 0.25 mm ID, film thickness 0.25 µm capillary column (HP-INNOWAX, Agilent Technologies). The gas carrier was helium (flow 1.5 mL/min) and the SPME injections were splitless (straight glass line, 0.75 mm ID) and performed at 240 °C for 20 min, during which the thermal desorption of analytes from the fiber occurred. The oven parameters were as follows: the initial temperature was 40 °C, which was held for 3 min, followed by an increase to 240 °C at a rate of 5 °C/min, and then held for 10 min. The

injector temperature was 240 °C. The mass spectrometer was operated in scan mode over a mass range from 33 to 300 amu (2 s/scan) at an ionization potential of 70 eV. The identification of volatile compounds was achieved by comparing the sample mass spectra with those in the Wiley library (Wiley7, NIST 05). The amounts of the individual compounds were expressed as a peak area percentage obtained by the automatic integration of the peak area of the compound/∑peak areas of all the compounds identified in the chromatograms. All the analyses were performed in duplicate. The operating conditions were in accordance with Di Renzo, Reale, Boscaino, & Messia (2018).

*2.10 Statistical analyses*

An analysis of variance (ANOVA) on the physico-chemical and colorimetric parameters included the kefir drink (KD) and kefir drink culture type (KD-CT) as the primary effects, and their interaction. The kefir drink had two levels: traditional kefir (TK) and backslopped kefir (BK); five different kefir drink culture types (KD-CT) were used, and they were coded as A, B, C, D, and E.

The data on the physico-chemical and colorimetric parameters were analyzed according to a split-block design, in which the two replications of the experiment were considered as blocks. The significance of the error variances for the primary effects (KD; KD-CT) were tested, and, in the event of a lack of significance, a pooled error variance was generated and used to test the primary effects and interaction variances. A mean comparison between the two kefir drink levels was performed by least significant difference (LSD) test, whereas multiple comparisons among the means were performed using the honest significant difference (HSD) test for the kefir drink culture type and the KD x KD-CT interaction. The same ANOVA model was applied to the microbiological plate count variables (LAB, AAB, and yeasts), but the kefir factor had three level samples (kefir grains-KG, traditional kefir-TK and backslopped kefir-BK) hereafter referred as KS. Multiple comparisons were performed by honest significant difference (HSD) test using JMP software (version 11.0).

Statistical analyses of the volatile compounds were performed using SYSTAT 13.0 for Windows (Systat Software Inc., Richmond, CA, USA). The data were expressed as the means ± standard error

of the mean. The statistical significance was evaluated by one-way analysis of variance (ANOVA) with Tukey's HSD test. A *p*-value of <0.05 was considered statistically significant. A principal component analysis (PCA) based on the contents of the volatile constituents was performed.

For the sequencing, the data sample coverage and diversity index (alpha diversity) were calculated using the *diversity* function in the vegan package (Dixon, 2007). For the 16S data, Unweight UniFrac distance matrices were used to find differences between samples by Anosim and Adonis statistical tests using the *vegan* function in the R environment. Non-parametric pairwise Wilcoxon tests were used as appropriate to determine the significant differences in the alpha diversity index, OTU abundance or VOC composition among the three types of samples. Principal component analyses (PCA) were performed and plotted using the *dudi.pca* function through the *made4* package of R. Pairwise Spearman's correlations between the taxa and volatile organic compounds were assessed with the R package *psych*. The P values were adjusted for multiple testing using the Benjamini-Hochberg procedure, which assesses the false discovery rate (FDR).

## 3. Results and Discussion

### *3.1 Characteristics of kefir grains from Bosnia and Herzegovina*

Macroscopic observations of the five milk kefir grains collected from Bosnia and Herzegovina showed that all of the grains were lobed and irregular in shape and they differed in their size and color (Supplementary Figure 2). In particular, the KGA and KGB grains were the largest, spanning approximately 4 cm; the KGC grain was the smallest (approximately 1 cm in length), while KGD and KGE were approximately 2 cm in length. The KGC grain also appeared whiter than the others, with a light yellow color. The KGE grain was characterized by a cavity at the center of the grain.

*3.2 Physico-chemical parameters, color profile of kefir drinks and microbial counts of kefir grains, and kefir drinks*

Concerning the statistical analysis of the data obtained from the physico-chemical and colorimetric profiles (Supplementary Table 1), the Blocks x KD and Blocks x KD-CT variances were always non-significant for almost all the variables. Therefore, a single pooled error variance was applied to test the primary effects and the interaction variances in the physico-chemical and colorimetric profile parameters.

The ANOVA results (Supplementary Table 1) showed that the kefir drink variance was significant for all the parameters except for fat and lightness (L), and the KD-CT variance highlighted significant differences in the pH, TTA, and D-lactic acid. Moreover, significant KD x KD-CT interaction was detected only for the pH and L-lactic acid.

A significant increase in the dry matter content for BKs (11.85%) compared to the TK products (10.88%) was recorded (Table 1). The same trend was observed for protein, lactose, and ash contents, with significantly higher values for BKs (3.35%, 2.92% and 0.70% respectively) than TKs (3.01%, 2.39% and 0.66%, respectively). The fat content of the BKs increased (3.79%) compared to the TKs (3.40%), even though this difference was not significant. Overall, the proximate compositions of the kefir drinks were very similar in terms of the dry matter, fat, protein, ash and lactose contents, when compared to the results noted by Sekkal-Taleb (2016) and reported by Irigoyen, Arana, Castiella, Torre, & Ibanez (2005) using traditional fermentation method and by Kim et al. (2018) using both traditional and backslopping methods. No significant differences in the protein, fat and ash contents between TK and BK were detected by Kim et al. (2018), although some other differences in the nutritional factors for TK and BK were found. Interestingly, in the present study, protein content results for TKs and BKs (3.01% and 3.35%, respectively) were lower than the ones reported by Satir & Seydim (2016) that applied the same fermentation methods to kefirs manufactured using milks from caprine and bovine species. As expected, the lactose content of TKs and BKs were lower than

that in the milk (4.65%), and this reduction rate was higher with respect to the values obtained by Irigoyen et al. (2005) and Garcia Fontan, Martinez, Franco, & Carballo (2006).

The highest pH values in the BK samples with respect to the TK samples were related to the minor TTA values in BK. An increase in the pH from TK to BK was also reported by Kim et al. (2018) who underlined that the higher pH would result in positive sensory features such as flavor and taste features over the shelf-life of the product (Kim et al., 2018). The pH value of BKs (4.30; Table 1) was the same as that reported by Kim et al. (2018) for the TK, and this value is indicated by Gao & Li (2016) as one of the best chemical characteristics of kefir. The D- and L-lactic acid contents showed opposite trends in TKs and BKs; the TK presented a significantly higher D-lactic acid content (5.79 g/L) than the BK (1.48 g/L), whereas a non-significant difference in L-lactic acid was observed between the BKs (5.20 g/L) and TKs (2.54 g/L). Certainly, the high amount of LAB (> 8.5 log CFU/mL) and AAB (≅ 9.0 log CFU/mL) counts (Table 2) in both kind of drink samples, strongly contributed to the acidification. The different acidification as well as the different lactose content between TKs and BKs was highly influenced by the different activity, proportions, interactions and utilization of lactose of the microbial starter cultures occurring in the different kefir drinks as highlighted below at paragraphs 3.3 and 3.4.

The comparison among the means of the five KD-CT showed that culture type B had a significantly higher pH level (4.26) then all the other cultures. Significant differences were also detected among the culture types for the TTA; culture type C had a significantly higher D-lactic acid content then all the other cultures except D, whereas the L-lactic acid content did not differ among the culture types (Table 1). For the remaining variables (compositional and colorimetric), the five culture types were very similar since no differences were detected (Table 1).

The pH and L-lactic acid content showed a significant interaction between the KD and KD-CT. For the pH parameters, multiple comparisons showed that the five culture types had significantly higher pH values in BKs than in TKs; however, culture type B revealed a much higher increase (3.88 in TK vs 4.63 in BK) than all the other cultures (Supplementary Table 2). The KD x KD-CT interaction in

L-lactic acid was primarily due to the significant increase in kefir produced with culture type E from traditional to backslopped kefir; all the other cultures showed a non-significant increase for this parameter (Supplementary Table 2).

For the colorimetric profile, the two kefir drinks did not differ for the lightness parameter (L), whereas a significantly lower redness index (a*) and significantly higher b* and chroma were found for BKs (Table 1 and Supplementary Table 1). The mean values for the L coordinate resulted in lower levels (86.08 and 85.49, respectively for TKs and BKs) compared to data reported by Gul, Atalar, Mortas, & Dervisoglu (2018) for kefirs produced from cow and buffalo milk (91.93 and 92.22) using kefir grains according to traditional methodology, and by Znamirowska et al. (2017) for natural sheep kefir (91.44). The red-green coordinate (a*) showed lower means (-7.90 and -8.48), therefore resulting in lightly greenish color perception compared with those reported by Gul et al. (2018) for cow and buffalo kefir (-1.01 and -1.49, respectively) and by Znamirowska et al. (2017) for sheep milk kefirs (-4.17). The yellow-blue coordinates (b*) were similar to the ones measured in commercial and traditional cow milk kefir (Gul et al., 2018). The saturation index values (chroma), which were significantly different between kefir products, were higher compared to the commercial and traditional cow milk kefir noted by Gul et al. (2018).

Concerning microbial counts, the ANOVA results for the LAB, AAB and yeast counts are reported in Supplementary Table 3, and they showed that all the sources of variation were highly significant. Multiple comparisons for kefir grains and drinks showed a significant increase in LAB and AAB from kefir grains (KGs) to TKs and from TKs to BKs. Unlike the LAB and AAB, the yeast counts decreased progressively and significantly from the grains to the BKs (Table 2). This trend is not surprising since Marshall & Cole (1985) reported that when kefir was used as a starter culture for subsequent fermentations, the balance in the microbiota of the deriving products was lost since the lactobacilli counts remained high but the yeast counts were progressively reduced. The yeast counts reduction during the backslopping process was also recently confirmed by Kim et al. (2018). These data can be explained by several studies that have reported that cells of yeasts are localized on the

outer areas of grains, and therefore they may fall into milk easily, reducing the rate of growth in milk, probably due to the absence of the symbiotic association with LAB and AAB that occurs within the grains (Garofalo et al., 2015; Guzel-Seydim et al., 2005; Kesmen & Kacmaz et al., 2011; Leite et al., 2013a; Pintado, Lopes da Silva, Fernandes, Malcata, & Hogg, 1996). Kim et al. (2018) underlined some positive effects from a commercial point of view that were related to the decreasing yeast counts since a reduced rate of low-temperature fermentation led by yeasts would prolong the shelf-life of the drink.

The overall LAB and yeast counts of kefir grains in the present study (7.99 log CFU/g and 7.62 log CFU/g, respectively; Table 2) were consistent with those generally reported in the literature, and they may vary from 4 to 9 log CFU/g and from 5 to 8 log CFU/g, respectively (Arslan, 2015; Garofalo et al., 2015; Guzel-Seydim et al., 2005; Kök Taş et al., 2012; Prado et al., 2015; Rea et al., 1996; Sarkar, 2008). The LAB counts significantly increased until they reached 8.75 log CFU/mL in TKs and 9.30 log CFU/mL in BKs, reaching values very similar to those reported by Kim et al. (2018). The TKs yeast counts were very similar to those reported by Guzel-Seydim et al. (2005) (6.16 log CFU/mL) and consistent with Arslan (2005), who reported values ranging from 3 to 6 log CFU/mL. Furthermore, Kim et al. (2018) also reported very similar yeast counts [7.10 log CFU/mL (TK) and 5.22 log CFU/mL (BK)] relative to those found during the present study [6.31 log CFU/mL (TKs) and 5.90 log CFU/mL (BKs)] (Table 2).

Concerning AAB, these microorganisms are usually considered as minority species within the kefir grain microbiota since they are only occasionally detected at counts from 4 to 6 log CFU/g (Dobson et al., 2011; Farnworth, 2005; Guzel-Seydim et al., 2011; Rea et al., 1996), whereas in the present study, the viable AAB counts were higher than 7 log CFU/g (Table 2). Furthermore, the AAB numbers significantly increased in the TKs and BKs, reaching values (8.98 log CFU/mL and 9.23 log CFU/mL, respectively; Table 2) that were higher than those reported in the literature for AAB in the TK (6 log CFU/mL; Irygoyen et al., 2005). However, it is of note that an underestimation or undetection of AAB by culture-dependent techniques is often possible due to limited growth of AAB

on synthetic media, thus highlighting the importance of employment of suitable isolation media coupled with culture-independent techniques (Bourrie et al., 2016; Garofalo et al., 2015). Although the presence of AAB in kefir is sometimes considered undesirable, it is well known that they play a crucial role in both the microbial consortium and the quality features of the final drink (Bourrie et al., 2016; Leite et al., 2013). The high AAB counts detected in the present study has certainly a role within the kefir microbiota thus influencing the volatile compounds profile of the related kefir drinks as discussed at paragraphs 3.3 and 3.4.

For the LAB, AAB and yeasts, significant differences were found among the five kefir drink culture types, as shown in Table 3. In particular, culture type C had the highest count levels for the three microbial parameters.

The interaction analysis revealed interesting differences in the trends for the five culture types, from the grains to the drinks (KS). The LAB counts increased from the KGs (7.79 – 8.44 log CFU/g) to the TKs (8.47 – 9.05 log CFU/mL) for all five cultures with different rates (Figure 1A), but similar final counts were detected in the BKs (9.07 – 9.38 log CFU/mL).

Figure 1B shows that in spite of the significant differences among the KGs, the five cultures reached similar AAB counts in the BKs (9.04 – 9.37 log CFU/mL).

A decrease in yeast counts (Figure 1C) was observed when moving from KGs (7.04 – 7.92 log CFU/g) to TKs (5.95 – 6.75 log CFU/mL) and then to BKs (5.16 – 6.37 log CFU/mL), but with a different trend for each culture. However, a significant difference between the TKs and BKs was detected only for culture type A.

*3.3 Microbiota in kefir grains and kefir drinks*

The bacterial diversity of the KGs and the kefir drinks (TKs and BKs) was obtained through rRNA gene amplicon sequencing. The total number of paired sequences obtained by 16S rRNA gene sequencing reached 456269 raw reads. After the data analysis, a total of 358468 reads passed filters

applied through QIIME, with a median value of 21362 ± 13.996 reads/sample and a mean sequence length of 465 bp. The rarefaction analysis and Good's coverage, as expressed as a median percentage (98%), indicated satisfactory coverage for all the samples. The alpha diversity index showed a higher OTUs richness in the TK and BK samples if compared with the KGs (FDR < 0.05). However no significant difference was observed between the TKs and BKs for all the indexes (Supplementary Figure 3).

The Adonis and analysis of similarity (ANOSIM) statistical tests based on the Unweight UniFrac distance matrix showed significant differences among the three type of samples ($P < 0.001$). Differences between samples were further demonstrated by principal coordinate analysis (PCoA) based on the Unweight UniFrac distance matrix (Supplementary Figure 4). The PCoA clearly showed a separation between the three types of samples (KGs vs BKs vs TKs).

As shown in Figure 2 panel A, we observed a very simple microbiota composition that was dominated by *Lb. kefiranofaciens*, which had median values of 92.72, 42.37 and 22.17% of the relative abundance in the kefir grains, TKs and BKs, respectively; *Acetobacter syzygii* at 4.22, 27.09 and 18.76% of the relative abundance in kefir grains, TKs and BKs, respectively; and *Lactococcus lactis* at 0.83, 16.75 and 50.06% of the relative abundance in kefir grains, TKs and BKs, respectively.

In addition, we observed the presence of minor OTUs such as *Leuconostoc pseudomesenteroides*, (0.03% median value in kefir grains reaching 10% median value in BKs), *Bacillus sporothermodurans* (0.09% median value in kefir grains reaching 10% median value in TKs), *Lactobacillus parakefiri* in both kefir grains and TKs (2%) and reaching less than 1% in the BKs and the few LAB spread throughout the samples.

*Lb. kefiranofaciens* and *Lactobacillus* spp. (0.22% median value of the relative abundance in kefir grains) were highly associated with kefir grain samples, *A. syzygii* and *B. sporothermodurans* were associated with TK samples, and *Lc. lactis* and *Leuc. pseudomesenteroides* were associated with BKs (FDR < 0.05) (Supplementary Figure 5).

Lactobacilli generally dominate the kefir grain microbiota and among them *Lb. kefiranofaciens* is a homofermentative species primarily involved in grain formation and the production of kefiran (Bengoa et al., 2018; Dertli & Çon, 2017; Garofalo et al., 2015; Hamet et al., 2013; Kotova, Cherdyntseva, & Netrusov, 2016; Leite et al., 2012; Plessas, Nouska, Mantzourani, Kourkoutas, & Alexopoulos, 2017; Wang et al., 2012). Kefiran is an EPS with excellent rheological properties that improve the viscosity of fermented milk and it can be used as a nutraceutical thanks to its several biological activities (Prado et al., 2015). Furthermore, *Lb. kefiranofaciens* strains have shown antimicrobial and anti-inflammatory activities (Bourrie et al., 2016). *Lb. kefiranofaciens* is commonly associated to *Lb. parakefiri* in kefir grains microbiota (Bourrie et al., 2016) as also found in the present study although, in this case, the latter species was detected with low relative abundance. The relative abundance of *Lb. kefiranofaciens* was reduced in the kefir drinks (TK and BK) in which other species prevailed. The species *A. syzygii* was found in low amounts in the kefir grains, becoming consistent in kefir drinks, primarily in TKs. This interesting species, which was found for the first time in Brazilian kefir grain by da Miguel, Cardoso, Lago, & Schwan (2010), was found to be capable of binding the mycotoxins in milk and reducing their gastrointestinal absorption (Taheur et al., 2017). Overall, among the AAB, *Acetobacter* is indicated as the most abundant genera within kefir grains with the ability to produce vitamin B that favors the growth of other microorganisms within the kefir grain consortium (Bengoa et al., 2018; Leite et al., 2013). The species *Lc. lactis* and *Leuc. pseudomesenteroides* were found to be significantly prevalent in the kefir drinks more often than in the kefir grains, as already found in other studies (Bourrie et al., 2016; Dobson et al., 2011; Gao & Zhang, 2019; Kesmen & Kacmaz, 2011; Korsak et al., 2015; Kotova et al., 2016; Leite et al., 2013b; Simova et al., 2002). Specifically, Gao and Zhang (2019) reported the same bacterial shift in the present study from *Lb. kefiranofaciens*, which was dominant among kefir grains from Tibet, to *Lc. lactis* and *Leuc. pseudomesenteroides* that increased their relative abundance in TK. These differences among the kefir grain microbiota and kefir drink microbiota have been explained by the different distributions of microorganisms in the grains. It has been reported that long bacilli (rod-

shaped bacilli o lactobacilli) are generally distributed on the inner layers of the grains, while cocci, as also observed for the yeasts, are generally localized on the outer portion of the grains and are characterized by their weak grain adhesion (Gao, Gu, Abdella, Ruan, & He, 2012; Guzel-Seydim et al., 2005; Jianzhong, Xiaoli, Hanhu, & Mingsheng, 2009; Magalhães, de Melo Pereira, Dias, & Schwan, 2010; Magalhães, de Melo Pereira, Campos, Dragone, & Schwan, 2011; Rea et al., 1996; Wang et al., 2012). Therefore, cocci may easily fall into milk, and, thanks to their strong ability to grow in milk, they became dominant in kefir drinks (Gao & Zhang, 2019). In particular, *Lc. lactis* is a key species in fermented milks with an important flavoring activity (Maoloni et al., 2020a) and as also observed within the present study, other authors documented that its level increases in fermented kefir respect grains and it further increases in kefir produced with kefir as an inoculum (Bourrie et al., 2016; Simova et al., 2002). Gao & Zhang (2019) indicated that *Leuc. pseudomesenteroides* is able to grow in association with lactococci, and it also plays an important role in producing aromatic and flavoring compounds within the final product. Moreover, the association of *Leuc. pseudomesenteroides* with BK samples may be explained by the higher pH value of these end-products respect to TKs since *Leuc. pseudomesenteroides* may be affected by the medium acidification (Cardinali et al., 2017).

The mycobiota characterization was obtained by sequencing the D1 domain of the 26S rRNA genes, and a total of 1378677 raw sequences were obtained; 1369182 of these sequences passed the quality filtering with a median length value of 389 bp. The rarefaction analysis and Good's coverage display a satisfactory coverage (99%) in all the samples. The alpha diversity index (Supplementary Figure 6) showed a higher level of complexity in the TK and BK samples when compared with kefir grains (FDR < 0.05). No significant differences were observed between TK and BK if compared with kefir grains (FDR < 0.05). A significant difference among the three types of samples (ANOSIM $P < 0.001$) was observed through PCA based on the OTU table abundance (Figure 3) in which the kefir grains were well separated from TKs and BKs and a slight separation was observed between the TKs and BKs. The core mycobiota consisted primarily of *Kazachstania unispora* (47.40, 12.69 and 32.31% in

kefir grains, TKs and BKs, respectively as a median value of the relative abundance), *Saccharomyces cerevisiae* (36.55, 14.78 and 39.24%), and *Kl. marxianus* (12.69, 52.78, and 7.04%) (Figure 2, panel B) although with some differences among samples. In particular, the kefir grains B, C and D were characterized by the prevalence of *K. unispora* species, whereas kefir grains A and E were characterized by the dominance of *S. cerevisiae*. All these yeast species were primarily isolated from kefir grains and drinks from around the world (Bengoa et al., 2018; Plessas et al., 2017; Prado et al., 2015). In all the kefir grains, *Kl. marxianus* was detected, and it became more consistent in TKs. Interestingly, the potential use of some kefir-derived strains of *Kl. marxianus* as probiotics has been proposed due to their anti-inflammatory, antioxidant and hypocholesterolemic activities (Bengoa et al., 2018; Bourrie et al., 2016). *Kl. marxianus* is a lactose-positive yeast typically associated to dairy products, kefir grains and kefir due to its ability to metabolize the lactose in milk as a carbon source, thus ensuring the formation of ethanol, $CO_2$ and the typical yeasty flavor of kefir (Bengoa et al., 2018; Cardinali et al., 2016; Leite et al., 2013a; Maoloni et al., 2020a,b; Vardjan, Mohar Lorbeg, Rogelj, & Čanžek Majhenič, 2013). By contrast, *S. cerevisiae*, *K. unispora*, *Torulaspora delbrueckii* and *Pichia fermentans* are non-lactose fermenting yeasts that may multiply in kefir grains and milk by using galactose and glucose that are released by the lactose hydrolysis caused by other bacteria and yeast species (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2013; Garofalo et al., 2015). Indeed, it should also be noted that TK samples display a higher abundance of *T. delbrueckii*, *P. fermentans*, *Pichia manshurica* and *Pichia orientalis* when compared with kefir grains and BK samples. *T. delbrueckii* has already been found in kefir grains from Slovenia (Vardjan et al., 2013). *Pichia* species are oxidative yeasts well adapted to the dairy environment since previously detected in dairy products and fermented milks (Maoloni et al., 2020a). To the authors' knowledge, *P. manshurica* and *P. orientalis* have never been detected in kefir grains and kefir drinks although *Pichia* spp. has been found with any regularity within kefir and the identified species was *P. fermentans* (Bourrie et al., 2016). Specifically, *P. fermentans* was identified in Taiwanese kefir (Wang, Chen, Liu, Lin, & Chen,

2008) and it has been reported as one of the biofilm producers involved during the initial stages of the small kefir granules formation (Prado et al., 2015).

By comparing the relative abundance of the mycobiota, it was observed that several minor OTUs characterized the different samples (Supplementary Figure 7; FDR < 0.05). In particular, *Alternaria tenuissima*, *Cladosporium cladosporioides* and *Malassezia* spp. were associated with BK samples, while *Kazachstania* spp. was associated with kefir grains and, as already indicated, *Kl. marxianus* with TKs (Supplementary Figure 7). To the authors' knowledge, the filamentous fungi *A. tenuissima* and *Cl. cladosporioides* have never been isolated from kefir grains and kefir drinks, while the genus *Malassezia* has been detected, albeit in low abundance, in just one kefir sample, by Marsh et al. (2013). *Alternaria* spp., *Cladosporium* spp. and specifically *Cl. cladosporioides* are airborne eumycetes deriving from structure of plants and are plant pathogens or endophytes considered as food contaminants (Osimani et al., 2017). Although sub-dominants, these microorganisms have been found within all the kefir grains under study and the related kefir products. Their presence could be explained by the fact that the kefir grains were obtained from private households and therefore derived from an uncontrolled environment. An accurate selection of the kefir grains as microbial starters as well as the application of hygiene practices during commercial kefir manufacturing is recommended.

*3.4 Volatile organic compounds (VOCs) in kefir drinks*

The SPME-GC/MS analysis allowed us to detect and to identify more than 60 volatile components in the TK and BK samples revealing a complex volatile profile of the kefir samples obtained. Peaks with an area at < 1% of the total peak areas and with no significant differences (ANOVA, Tukey's HSD test) under the different conditions were discarded from further statistical and graphical analyses. Table 4 shows the 31 volatile components that primarily ($P < 0.05$) (ANOVA) characterized the samples. The most characteristic volatile compounds belonged to the ketones, esters and acetates, alcohols and acids and they were similar to the previous findings reported by Dertli & Çon (2017) for

four different traditional kefir samples. Among the carboxylic acids, acetic acid was the most representative one in all the fermented drinks (Table 4) probably due to the high AAB counts found in TK and BK samples. Upon determining the volatilome composition among the TK and BK samples, a separation was clearly observed between the types of samples based on the VOC composition, highlighting that the different methods used to produce the kefir drinks (TK and BK) significantly influenced the final flavor of the products. Furthermore, some differences in the volatile profile within each kefir drink culture type (A, B, C, D, E), as already shown for some physico-chemical parameters, microbial counts and microbial structure were also found (Table 4).

By comparing the presence of the VOCs among the samples, we observed the predominance (FDR < 0.05) of ethyl acetate, ethyl lactate, benzoic acid and ethanol in TKs. The presence of ethanol undoubtedly gives the desired flavor of a light alcoholic beverage to the final products, and higher alcohol contents may be associated with a slight yeasty flavor (Güzel-Seydim, Seydim, Greene, & Bodine, 2000). Ethanol is one of the main metabolites of the alcoholic fermentation produced by yeasts during the manufacturing of a self-carbonated, acidic and low-alcohol fermented milk beverage as kefir (Leite et al., 2013a). The significant reduction of this key metabolite of kefir in BKs (Table 4) was probably linked to the significant reduction of yeast counts among BK samples (Table 2). The BK samples displayed the 2-propanone, 2-butanone, acetoin, butanoic and hexanoic acids (Figure 4; FDR < 0.05) that are characteristic volatile components associated with a yogurt-like aroma or sweet cheese flavor (Dertli & Çon, 2017). Acetoin was also reported by Guzel-Seydim et al. (2000) and Güzel-Seydim, Seydim, & Greene (2000) as one of the major end products of microbial fermentation that characterizes the kefir aroma. More specifically, the hierarchical cluster analysis clearly showed this separation (Supplementary Figure 8). Indeed, TKs were primarily characterized by the presence of short chain fatty acid (e.g., acetic, benzoic, and, propanoic acids), different acetates such as ethyl acetate, ethyl lactate, and phenethyl acetate, and ketones (2,3-butanedione, 2-heptanone) and ethanol. In addition, isoamyl alcohol, 2,6-dimethyl-4-heptanol and phenethyl alcohol were characteristic of the TK samples. However, the BK samples predominantly showed medium and long chain fatty acid

(pentanoic, butanoic, hexanoic, heptanoic, octanoic and decanoic acids) and ketone compounds (acetoin and 2-nonanone).

By plotting the correlation between VOCs and microbiota (Figure 5; FDR < 0.05), we observed that *A. syzygii* displayed a significant positive correlation with 2,3-butanedione (diacetyl) and 2-heptanol while *B. sporothermodurans* was correlated with ethyl lactate, and both these bacteria were associated with TK samples. Diacetyl and 2-heptanol are two important flavoring agents of the dairy fermented food products (Reale et al., 2016) that are also responsible for the buttery flavor as well as caramel and fruity odor in red wine vinegar (Yu et al., 2012). The production of secondary alcohols such as 2-heptanol can be achieved from 2-heptanone a ketone also found among TK and BK samples (Mota-Gutierrez et al., 2019). The main OTU of the BK samples was *Lc. lactis*, which was correlated with 2-butanone, acetoin, isoamyl alcohol, 2,6-dimethyl-4-heptanol and phenethyl alcohol, while *Leuc. pseudomesenteroides* was correlated with formic, heptanoic, octanoic, nonanoic, decanoic and dodecanoic acids (Figure 5; FDR < 0.05). *Lb. kefiranofaciens* was highly correlated with ethyl acetate, ethanol, ethyl lactate and benzoic acid. Few correlations were observed between the VOCs and mycobiota; however, there was a notable correlation between *Kl. marxianus*, the more abundant yeast in the TK samples, with ethyl acetate, ethanol, ethyl lactate, benzoic acid and phenethyl acetate; these are volatile compounds that strongly characterized the TK samples (Figure 5). Additionally, among the yeasts, *S. cerevisiae* was associated with pentanoic acid and *T. delbrueckii* was associated with phenethyl alcohol (Figure 5; FDR<0.05). It is also interesting to note that, as recently reviewed by Mota-Gutierrez, Barbosa-Pereira, Ferrocino, & Cocolin (2019), VOCs are organic compounds that are naturally produced by microorganisms during fermentations as secondary metabolites, and they may also provide health benefits to consumers in addition to their sensory properties. In kefir, several health promoting features are ascribed to this non-microbial fraction composed by organic acids (as lactate, acetate, propionate and butyrate), short chain fatty acids, ethanol, diacetyl, acetaldehyde, esters, alcohol compounds, acetoin (Bengoa et al., 2018). In particular, among the VOCs detected in TKs, phenethyl alcohol (or 2-phenylethanol) confers a floral, rose-like odor, and it has been

demonstrated to have the ability to inhibit the growth of gram-negative bacteria and filamentous fungi (Mota-Gutierrez et al., 2019). The synthesis of phenethyl alcohol is performed by metabolism of yeasts, primarily by *Kl. marxianus*, *Pichia anomala*, *Pichia farinosa*, *Pichia kudriavzevii*, *S. cerevisiae* and *Wickerhamomyces anomalus* (Mota-Gutierrez et al., 2019). In addition, the ester phenethyl acetate (or 2-phenylethyl acetate) that is well known for its antimicrobial activity is derived from the transformation of amino acids that are metabolized by yeast species including *Kl. marxianus*, *Kluyveromyces lactis*, *P. anomala*, *P. farinosa*, *P. kudriavzevii* and *S. cerevisiae* (Mota-Gutierrez et al., 2019). Antimicrobial properties of kefir are also related to lactic and acetic acid, ethanol and diacetyl that are able to reduce pathogenic and spoilage microorganisms in kefir and in gastrointestinal and vaginal infections (Farnworth, 2005; Prado et al., 2015). Regarding the production of lactic acid, a high correlation was found among D-lactic acid and *Lb. kefiranofaciens*, *Kl. marxianus*, *Kluyveromyces* spp. and *Lactobacillus* spp. Specifically, *Lactobacillus* spp. and *Lb. kefiranofaciens* were more abundant in the TKs than the BKs and in the TKs the amount of D-lactic acid (5.79 g/L) was significantly higher with respect to the BK samples (1.48 g/L) (Table 1). The species *Lb. kefiranofaciens*, as well as other homofermentative species of *Lactobacillus* spp., in fact, can produce DL-lactic acid homofermentatively but with a marked excess of D-lactic acid (Fujisawa T., Adachi, Toba, Arihara, & Mitsuoka, 1988). Unlike the TK samples, the BK samples were characterized by high L-lactic acid (5.20 g/L) and low D-lactic acid (1.48 g/L) content (Table 1), highlighting that the backslopping method could move the ratio of two stereoisomeric forms to favor L-(+)-lactate. The dominance of *Lc. lactis* in the BK samples was probably responsible for the accumulation of L-lactic acid, since it is known that *Lactococcus* species have a homo-fermentative metabolism and produce exclusively L-lactic acid (Roissart & Luquet, 1994). Furthermore, the yeasts as *K. unispora* and *S. cerevisiae*, found in high abundance in BK samples, may also have contributed to L-lactic production (Whiting, 1976). This fact is favorable because the steroisomer D-lactic acid is at times harmful to human metabolism and can result in acidosis and decalcification (Hofvendahl & Hahn-Hägerdal, 2000).

The results related to kefir VOCs composition provide evidence that different processing conditions such as traditional and backslopping methods, influence the volatile compounds pattern related to the microbial composition developed, thus affecting the quality characteristics of the final products as confirmed by the Principal Component Analysis (PCA) (Figure 6). The first two principal components (PC) explained 70.7% of the total variance. Samples produced by traditional method (TKs) were clearly separated from samples obtained by backslopping (BKs) on the basis of their odors reported in Table 4. Based on flavornet and pherobase online databases the TK samples were primarily correlated with alcoholic (solvent), fruity, fatty and acid odors, whereas all the BK samples, with the exception of the BKB, were correlated with cheesy, buttery, floral and fermented odors. To the author's knowledge, this is the first report on volatilome profile of the backslopped kefir.

## 5. Conclusion

The five kefir grains collected in Bosnia and Herzegovina were dominated by lactobacilli, particularly *Lb. kefiranofaciens*. A shift in the bacterial species toward *A. syzygii*, *Lc. lactis* and *Leuc. pseudomesenteroides* from kefir grains to traditional kefir and backslopped kefir was then generally observed. In fact, the bacterial species that were detected with higher relative abundance in kefir grains were progressively reduced in kefir drinks, while the species that were minorities in the grains became dominant in the kefir drinks. The core mycobiota of the kefir grains under study were primarily characterized by the presence of *K. unispora*, *S. cerevisiae* and *Kl. marxianus*. Despite some differences among the five kefir drink culture types, the dominant mycobiota of backslopped kefir samples remained quite similar to that of the kefir grain samples, but unlike the LAB and AAB, the yeast counts decreased progressively from the grains to the backslopped kefir. The microbial dynamics observed here may be explained by the different localization and adhesion to grains by the microbial species involved in the grain microbiota as well as to their different growth abilities in milk.

Similar to the diversity of microbiota in the traditional and backslopped kefir samples, there were differences in some physico-chemical parameters and in the volatile components among kefir drinks. The backslopped method enhanced the final drink for protein, lactose and ash content, compared to traditional kefir, coupled with a optimal pH that contributes to a pleasant sensory profile. Most of the backslopped kefir samples were correlated with cheesy, buttery, floral and fermented odors, whereas the traditional kefir samples were correlated with alcoholic, fruity, fatty and acid odors. Overall, the data obtained in the present study provided evidence that different kefir production methods (traditional vs backslopping) influenced both the microbial composition and the volatile compounds pattern, as well as some physico-chemical parameters of the final product, thus affecting its quality characteristics. The dominance of *Lc. lactis* (that is a typical species of fermented milks) coupled with a progressive reduction of *Lb. kefiranofaciens* (the key microorganism of kefir) in backslopped kefir samples, as well as the yeast counts decrease, the ethanol reduction and lower acidity of the final product, prompted us to hypothesize that the backslopping method may have reduced some of the functional traits of kefir. Hence, the functional traits of backslopped kefir should be further established in order to verify the usefulness of a potential scale-up methodology for backslopping.

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**FIGURE CAPTIONS**

**Figure 1**. KS x KD-CT interaction: lactic acid bacteria (Panel A), acetic acid bacteria (Panel B) and yeast (Panel C) counts in kefir grains (log CFU/g), traditional kefir (log CFU/mL) and backslopped kefir (log CFU/mL) with the 5 culture types (CT). Means within each panel with different letters are different at $P < 0.05$. KS = kefir grains and kefir drinks; KD-CT = kefir drink culture type.

**Figure 2. Panel A)** The incidence of the major taxonomic groups detected by 16S rRNA gene sequencing. **Panel B)** Incidence of the major taxonomic groups detected by 26S rRNA gene sequencing. Only OTUs with an incidence above 0.2% in at least two samples are shown. KGA-KGE, kefir grain; TKA-TKE, traditional kefir; and BKA-BKE, backslopped kefir.

**Figure 3.** PCA based on the OTU abundance of 26S datasets as a function of the sample type. The first component (horizontal) accounts for 40.66% of the variance, and the second component (vertical) accounts for 22.53%.

**Figure 4.** Box plots showing the relative abundance of the VOCs indicate their differential abundance based on a pairwise Wilcoxon test (FDR $\leq 0.05$) in traditional kefir (blue bars) and backslopped kefir (green bars).

**Figure 5.** Correlation between the abundance of VOCs (mg/kg) and OTUs occurring at 0.2% in at least two samples. The intensity of the colors represents the degree of correlation between OTUs and VOCs as measured by the Spearman's correlations, where the color blue represents a positive degree of correlation and red a negative correlation.

**Figure 6**. Score and loading plots of the first and second principal components after the principal component analysis based on odors that primarily ($P < 0.05$) differentiated the traditional kefir (TKA-TKE) from the backslopped kefir (BKA-BKE). The odors related to the volatile organic compounds used in the PCA are listed in Table 4.

**Figure 1**.

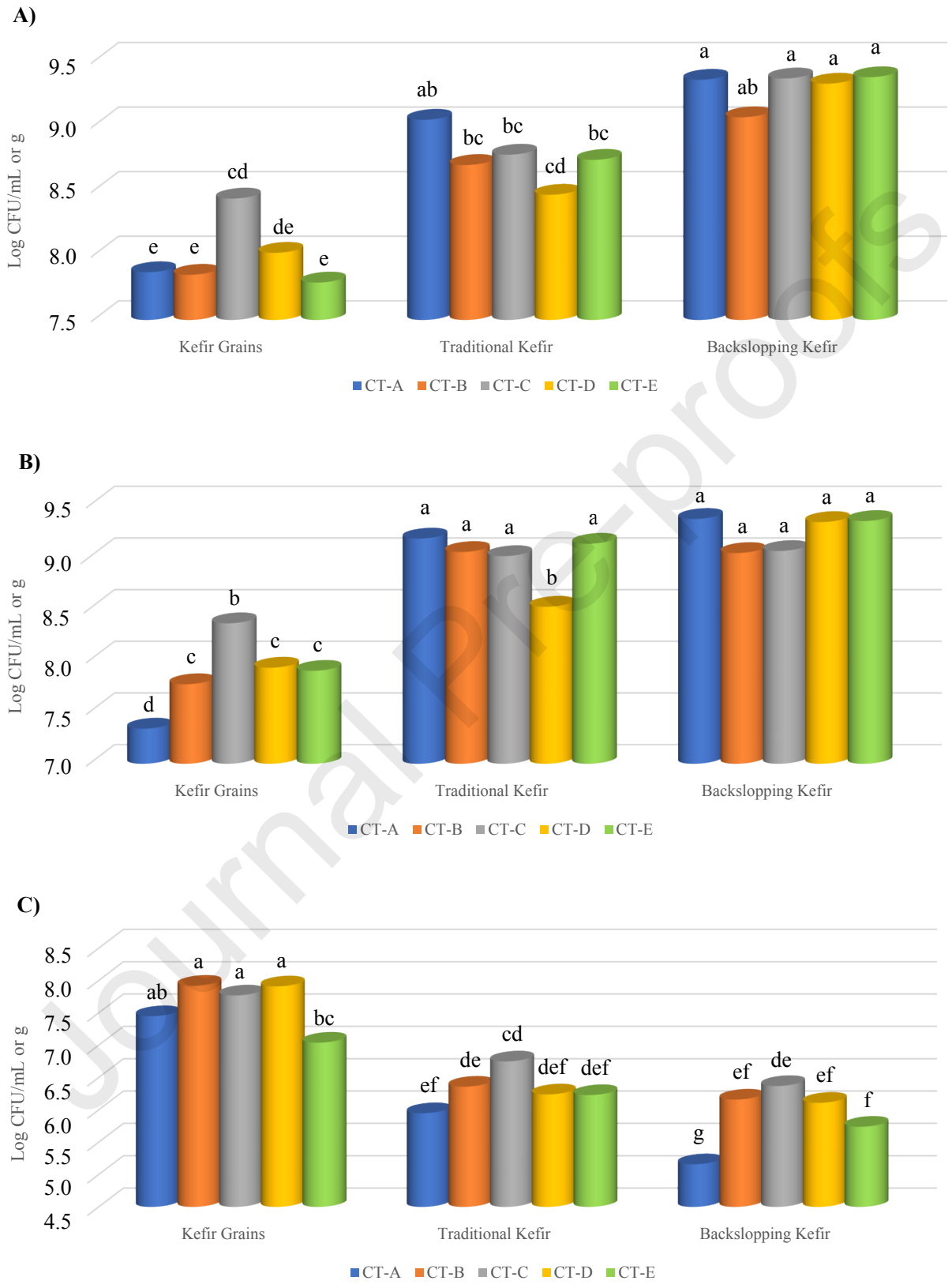
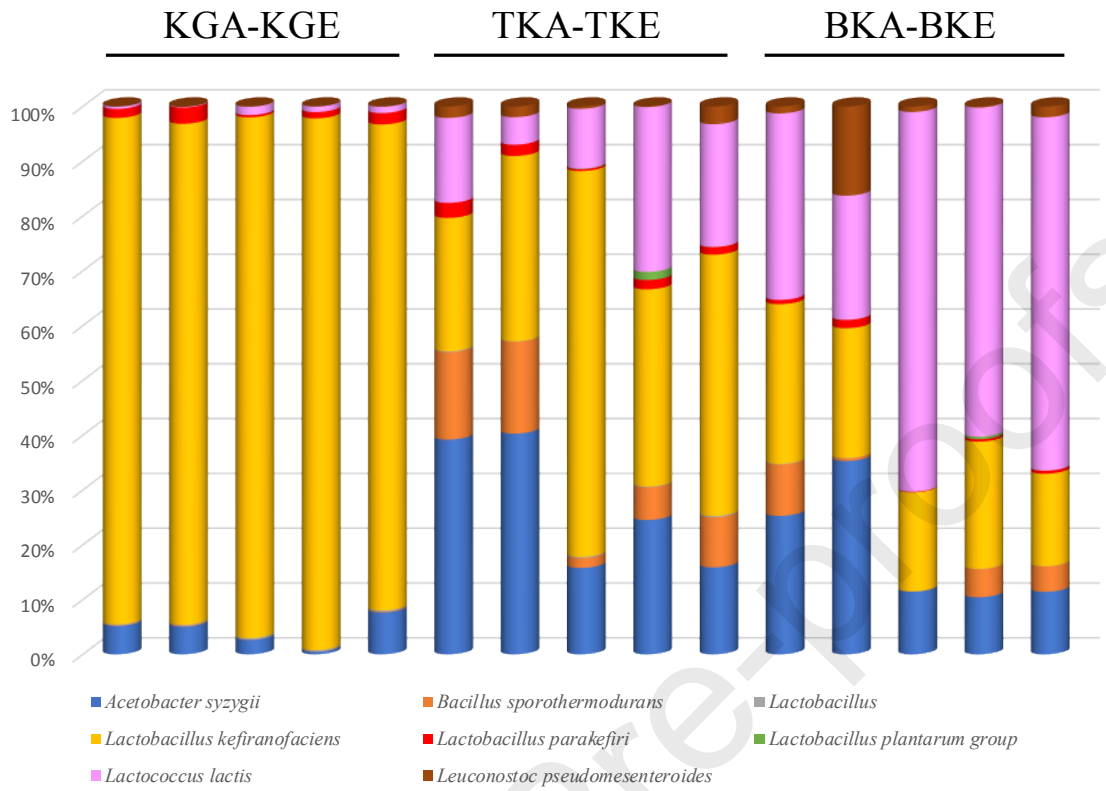


**Figure 2**

**A)**




**B)**

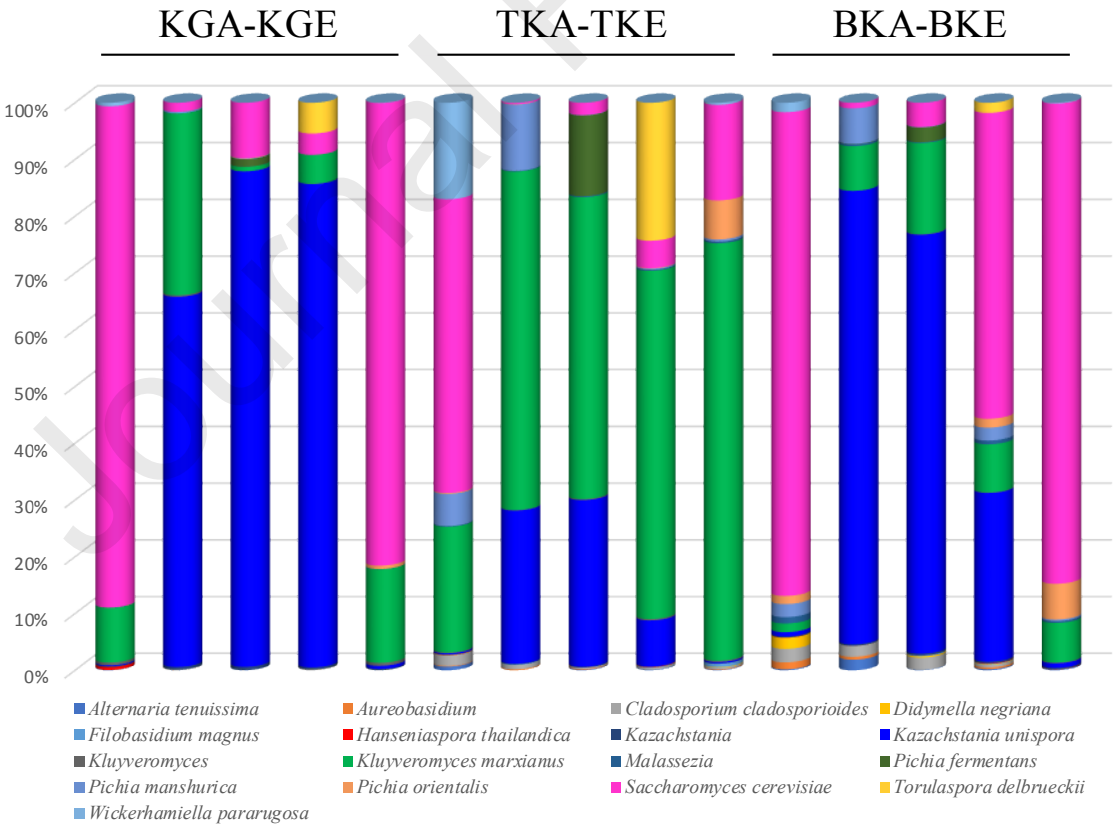


**Figure 3**

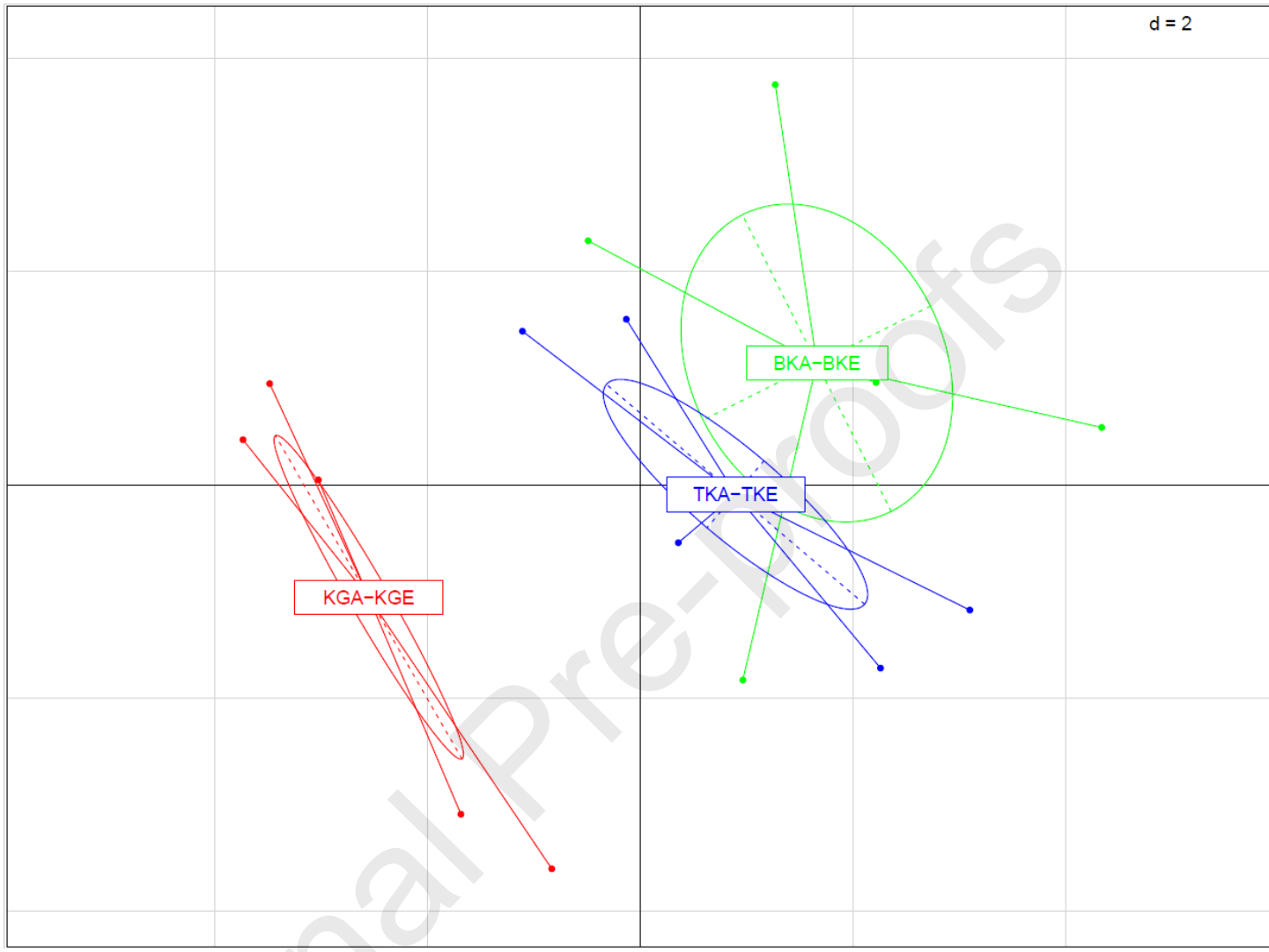


**Figure 4**

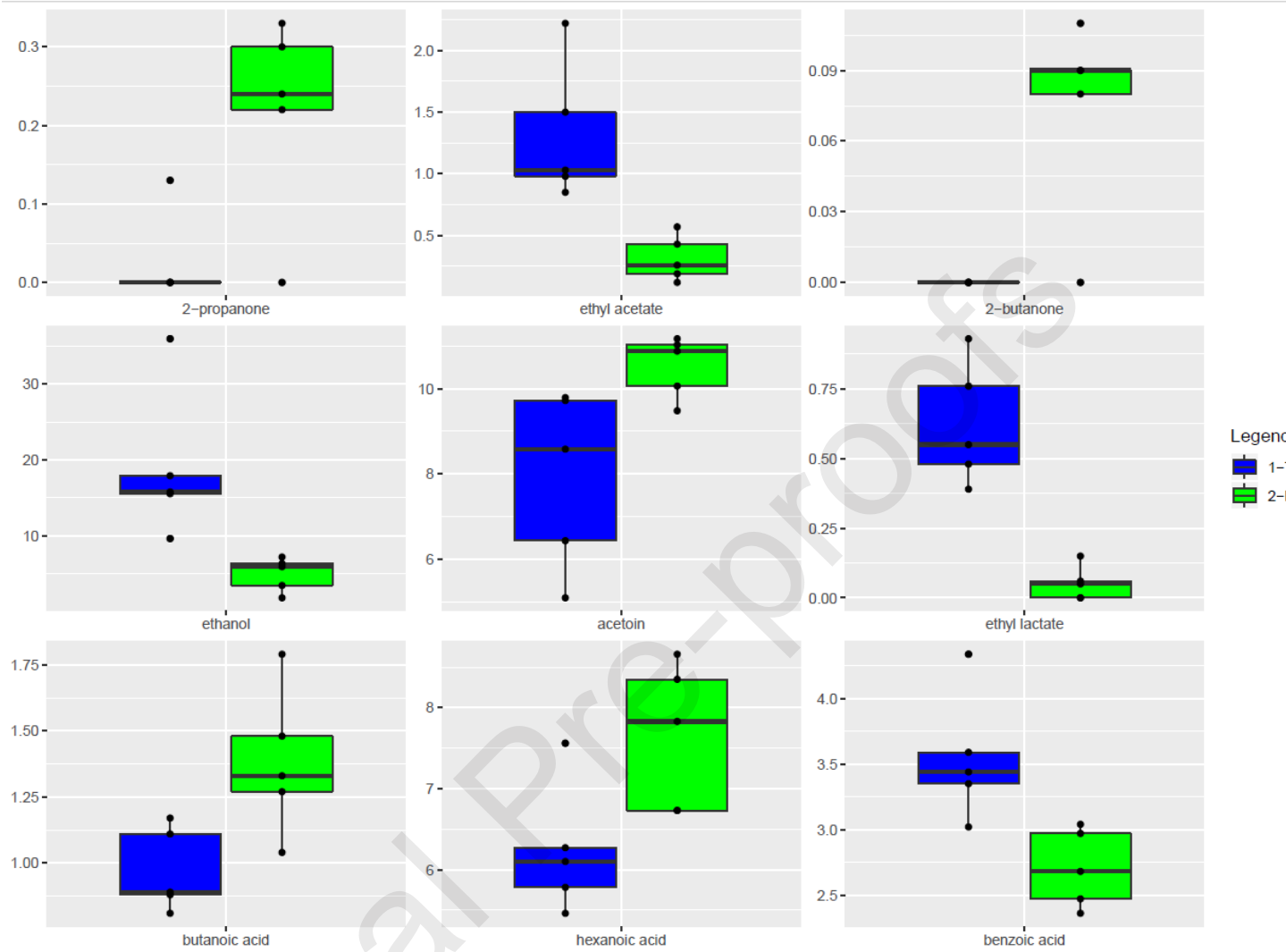
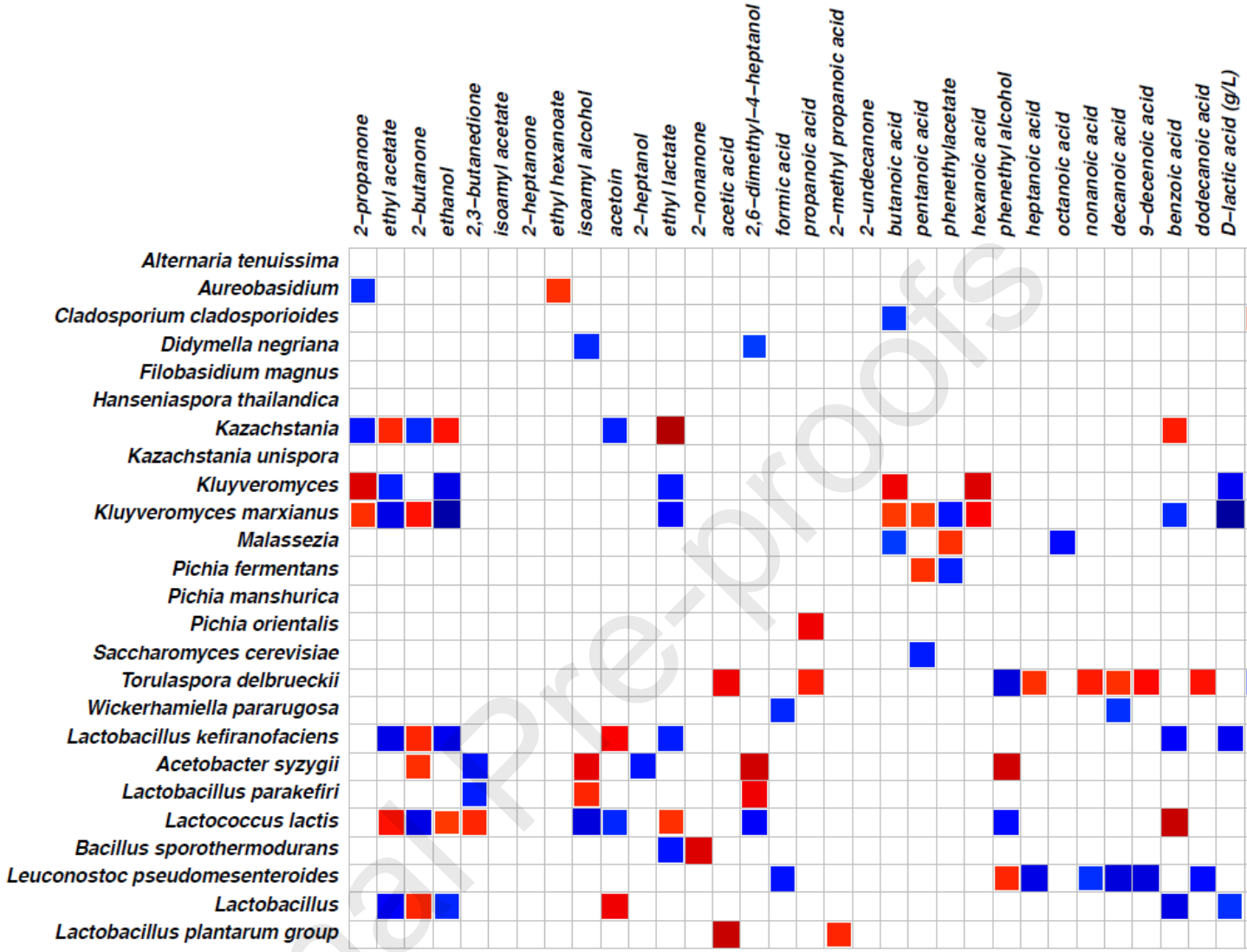


**Figure 5**



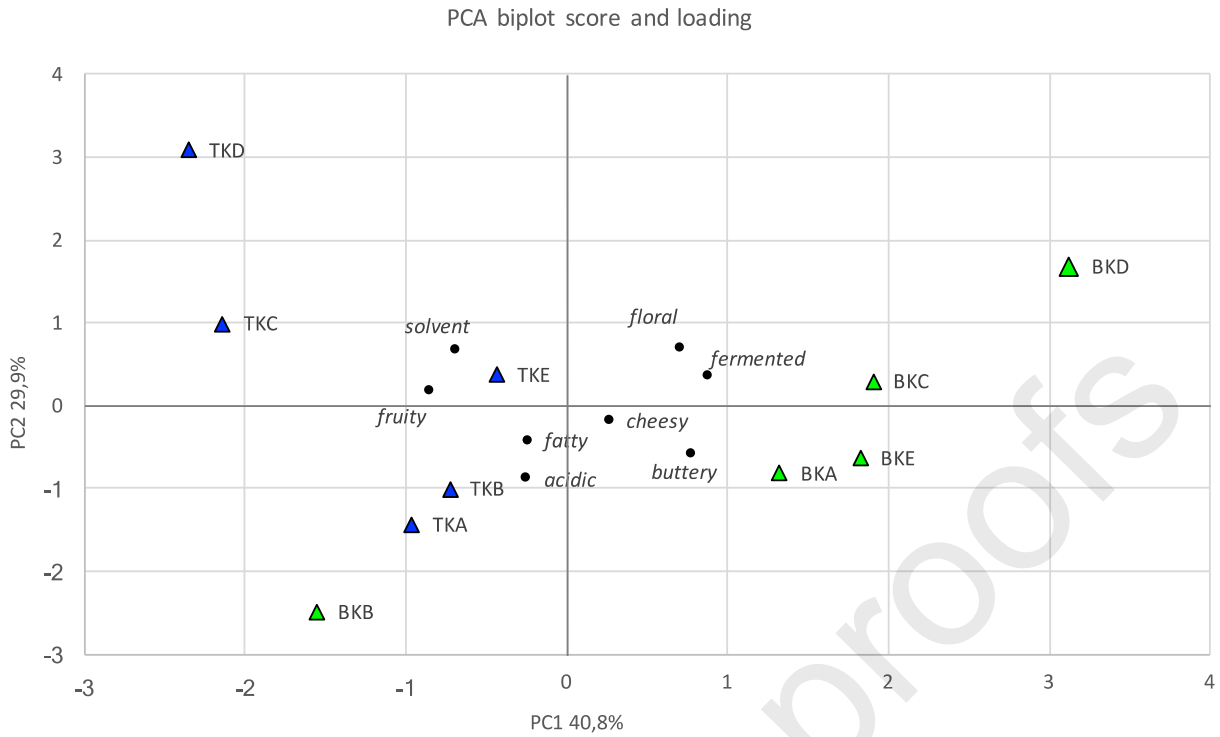



**Figure 6**

**Table 1** Physico-chemical and colorimetric parameters mean comparisons of kefir products using least significant difference (LSD) test and five kefir culture types using the honest significant difference (HSD) test

| | $H_2O$ (%) | D.M. (%) | Fat (%) | Protein (%) | Lactose (%) | Ash (%) | pH | TTA (g/L) | D-lactic acid (g/L) | L-lactic acid (g/L) | L | a* | b* | Chroma |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Kefir products** | | | | | | | | | | | | | | |
| **TK** | 89.11$^{a}$ | 10.88$^{b}$ | 3.40$^{a}$ | 3.01$^{b}$ | 2.39$^{b}$ | 0.66$^{b}$ | 3.74$^{b}$ | 11.42$^{a}$ | 5.79$^{a}$ | 2.54$^{a}$ | 86.08$^{a}$ | -7.90$^{a}$ | 9.10$^{b}$ | 12.07$^{b}$ |
| **BK** | 88.15$^{b}$ | 11.85$^{a}$ | 3.79$^{a}$ | 3.35$^{a}$ | 2.92$^{a}$ | 0.70$^{a}$ | 4.30$^{a}$ | 6.82$^{b}$ | 1.48$^{b}$ | 5.20$^{a}$ | 85.49$^{a}$ | -8.48$^{b}$ | 11.26$^{a}$ | 14.11$^{a}$ |
| **Culture types** | | | | | | | | | | | | | | |
| **KD-CT A** | 88.40$^{a}$ | 11.60$^{a}$ | 3.85$^{a}$ | 3.33$^{a}$ | 2.45$^{a}$ | 0.70$^{a}$ | 4.03$^{b}$ | 8.65$^{ab}$ | 2.90$^{b}$ | 4.42$^{a}$ | 85.90$^{a}$ | -8.17$^{a}$ | 10.46$^{a}$ | 13.29$^{a}$ |
| **KD-CT B** | 88.24$^{a}$ | 11.76$^{a}$ | 3.73$^{a}$ | 3.30$^{a}$ | 2.87$^{a}$ | 0.67$^{a}$ | 4.26$^{a}$ | 8.13$^{b}$ | 3.22$^{b}$ | 3.89$^{a}$ | 84.88$^{a}$ | -8.46$^{a}$ | 10.48$^{a}$ | 13.47$^{a}$ |
| **KD-CT C** | 88.64$^{a}$ | 11.37$^{a}$ | 3.59$^{a}$ | 3.08$^{a}$ | 2.41$^{a}$ | 0.69$^{a}$ | 3.89$^{c}$ | 10.01$^{a}$ | 4.86$^{a}$ | 3.42$^{a}$ | 86.12$^{a}$ | -8.22$^{a}$ | 10.37$^{a}$ | 13.24$^{a}$ |
| **KD-CT D** | 89.28$^{a}$ | 10.72$^{a}$ | 3.28$^{a}$ | 2.92$^{a}$ | 2.74$^{a}$ | 0.69$^{a}$ | 3.99$^{bc}$ | 8.93$^{ab}$ | 3.91$^{ab}$ | 3.14$^{a}$ | 86.27$^{a}$ | -8.18$^{a}$ | 9.63$^{a}$ | 12.67$^{a}$ |
| **KD-CT E** | 88.62$^{a}$ | 11.38$^{a}$ | 3.54$^{a}$ | 3.28$^{a}$ | 2.79$^{a}$ | 0.68$^{a}$ | 3.95$^{bc}$ | 9.88$^{a}$ | 3.28$^{b}$ | 4.49$^{a}$ | 85.75$^{a}$ | -7.94$^{a}$ | 9.97$^{a}$ | 12.77$^{a}$ |

Means within the same column with different letters for kefir products are different at $P \leq 0.05$
Means within the same column with different letters for culture types are different at $P \leq 0.05$
D.M. = dry matter, TTA = Total Titratable Acidity, L = lightness, a* = redness, b* = yellowness, TK = traditional kefir, BK = backslopped kefir, KD-CT = kefir drink culture type

**Table 2** Lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeast counts mean comparisons using the honest significant difference (HSD) test of kefir grains and drinks

| | LAB | AAB | Yeasts |
|---|---|---|---|
| **KG** | 7.99±0.29 [c] | 7.86±0.37 [c] | 7.62±0.36 [a] |
| **TK** | 8.75±0.20 [b] | 8.98±0.26 [b] | 6.31±0.28 [b] |
| **BK** | 9.30±0.13 [a] | 9.23±0.16 [a] | 5.90±0.47 [c] |

Means within the same column with different letters are different at $P \leq 0.05$
KG = kefir grains, TK = traditional kefir, BK = backslopped kefir
Viable counts are expressed as log CFU/g for KG; viable counts are expressed as log CFU/mL for TK and BK

**Table 3** Lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeast counts mean comparisons using the honest significant difference (HSD) test of kefir drink culture type (KD-CT)

| | LAB (log CFU/mL) | AAB (log CFU/mL) | Yeasts (log CFU/mL) |
|---|---|---|---|
| **KD-CT A** | 8.76$^{ab}$ | 8.63$^{ab}$ | 6.19$^{b}$ |
| **KD-CT B** | 8.54$^{b}$ | 8.62$^{ab}$ | 6.81$^{a}$ |
| **KD-CT C** | 8.86$^{a}$ | 8.81$^{a}$ | 6.96$^{a}$ |
| **KD-CT D** | 8.61$^{b}$ | 8.59$^{b}$ | 6.75$^{a}$ |
| **KD-CT E** | 8.64$^{ab}$ | 8.79$^{a}$ | 6.33$^{b}$ |

Means within the same column with different letters are different at $P \leq 0.05$

**Table 4** Volatile organic compounds (VOCs) identified by solid phase microextraction/gas chromatography-mass spectrometry in the five traditional and backslopped kefir samples

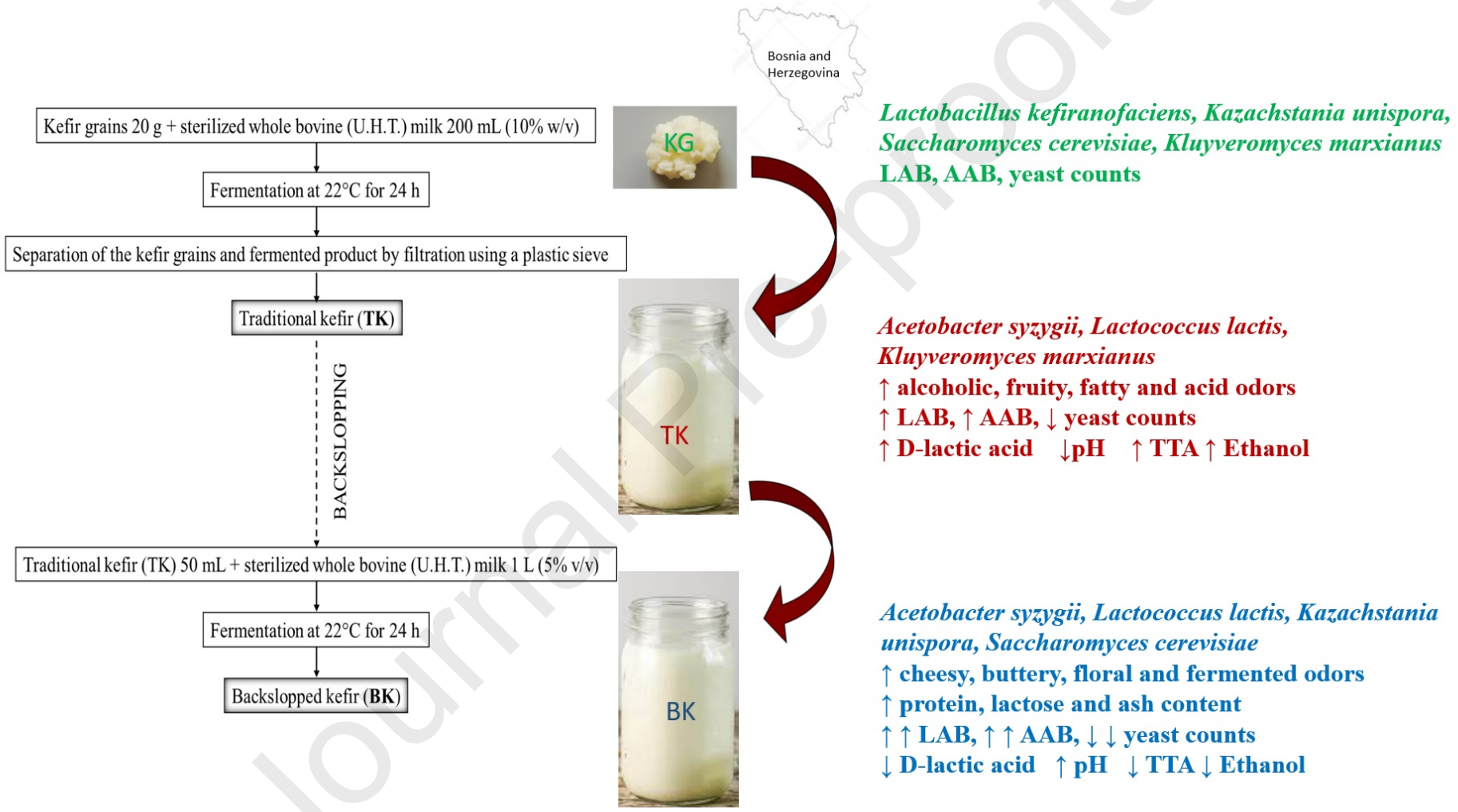
| | | Traditional kefir | | | | | Backslopped kefir | | | | | Odor* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **RI** | **Compounds** | **A** | **B** | **C** | **D** | **E** | **A** | **B** | **C** | **D** | **E** | |
| | **Ketons** | | | | | | | | | | | |
| 774 | 2-propanone | nd | nd | 0.1±0.0 | nd | nd | 0.5±0.1$^{a}$ | 0.3±0.1$^{b}$ | 0.2±0.0$^{b}$ | 0.2±0.0$^{b}$ | nd | solvent, ethereal |
| 924 | 2-butanone | nd | nd | nd | nd | nd | 0.1±0.0$^{a}$ | nd | nd | 0.1±0.0$^{a}$ | 0.1±0.0$^{a}$ | buttery, yogurt-like aroma |
| 1008 | 2,3-butanedione | 1.9±0.2$^{a}$* | 1.1±0.2$^{b}$* | 1.4±0.2$^{b}$* | 1.9±0.3$^{a}$* | 0.7±0.0$^{c}$ | 0.7±0.1$^{a}$§ | 1.5±0.0$^{b}$§ | 0.6±0.1$^{a}$§ | 0.7±0.1$^{a}$ | 0.5±0.1$^{a}$ | buttery, sweet, creamy |
| 1158 | 2-heptanone | 1.1±0.1$^{a}$ | 4.4±0.4$^{b}$* | 3.4±0.1$^{c}$* | 1.0±0.1$^{a}$* | 2.8±0.1$^{d}$* | 1.2±0.1$^{a}$ | 0.9±0.1$^{a}$§ | 1.0±0.1$^{a}$§ | 2.8±0.1$^{b}$§ | 1.9±0.2$^{c}$§ | cheesy |
| 1275 | acetoin | 8.6±0.4$^{a}$* | 9.8±1.3$^{a}$ | 6.4±0.5$^{b}$* | 5.1±0.8$^{b}$* | 9.7±0.4$^{a}$* | 10.1±0.5$^{a}$§ | 9.5±1.3$^{a}$ | 11.0±1.3$^{a}$§ | 10.9±0.6$^{a}$§ | 11.2±0.5$^{a}$§ | buttery, creamy, dairy |
| 1390 | 2-nonanone | 1.3±0.1$^{a}$* | 1.0±0.1$^{b}$* | 2.0±0.2$^{c}$* | 1.5±0.1$^{a}$ | 1.4±0.1$^{a}$ | 1.7±0.2$^{a}$§ | 1.6±0.2$^{a}$§ | 1.4±0.0$^{a}$§ | 1.4±0.0$^{a}$ | 1.5±0.2$^{a}$ | cheesy, sweet |
| 1598 | 2-undecanone | 0.4±0.0$^{a}$ | 0.5±0.0$^{a}$* | 0.5±0.0$^{a}$ | 0.3±0.0$^{a}$ | 0.4±0.0$^{a}$ | 0.4±0.1$^{a}$ | 0.6±0.0$^{b}$§ | 0.4±0.1$^{a}$ | 0.5±0.0$^{a}$ | 0.5±0.1$^{ab}$ | fruity, creamy |
| | **Esters and acetates** | | | | | | | | | | | |
| 910 | ethyl acetate | 1.0±0.1$^{a}$* | 0.9±0.1$^{a}$* | 2.2±0.2$^{b}$* | 1.5±0.1$^{c}$* | 1.0±0.1$^{a}$* | 0.2±0.0$^{a}$§ | 0.6±0.0$^{b}$§ | 0.4±0.1$^{c}$§ | 0.3±0.0$^{c}$§ | 0.1±0.0$^{a}$§ | fruity, sweet |
| 1116 | isoamyl acetate | nd | nd | nd | nd | nd | nd | nd | nd | 0.1±0.0$^{a}$ | nd | fruity, banana |
| 1188 | ethyl hexanoate | 0.2±0.0$^{a}$ | nd | nd | 0.2±0.0$^{a}$ | nd | nd | nd | 0.0±0.0$^{a}$ | nd | nd | fruity, waxy, green banana |
| 1298 | ethyl lactate | 0.5±0.0$^{a}$* | 0.9±0.1$^{b}$ | 0.4±0.1$^{a}$* | 0.8±0.1$^{b}$ | 0.6±0.1$^{a}$* | 0.1±0.0$^{a}$§ | nd | 0.1±0.0$^{a}$§ | nd | 0.1±0.0$^{a}$§ | fruity, buttery, butterscotch |
| 1799 | phenethylacetate | 0.3±0.0$^{a}$ | 0.6±0.0$^{b}$ | 3.6±0.0$^{c}$* | 0.8±0.0$^{d}$* | 0.6±0.1$^{b}$ | nd | nd | 0.9±0.1 $^{a}$§ | 0.1±0.0 $^{a}$§ | nd | fruity, sweet, honey |
| | **Alcohols** | | | | | | | | | | | |
| 957 | Ethanol | 9.7±1.2$^{a}$* | 15.5±0.5$^{b}$* | 17.9±1.4$^{b}$* | 35.9±4.4$^{c}$* | 15.8±2.5$^{b}$* | 1.9±0.2$^{a}$§ | 6.4±1.0$^{bc}$§ | 5.9±0.1$^{b}$§ | 7.2±0.1$^{c}$§ | 3.5±0.5$^{d}$§ | solvent, ethereal, |
| 1210 | isoamyl alcohol | 4.5±0.5$^{a}$* | 3.4±0.5$^{b}$* | 5.2±0.4$^{a}$* | 7.6±1.1$^{c}$* | 6.7±0.6$^{c}$* | 8.9±1.1$^{a}$§ | 1.7±0.2$^{b}$§ | 10.3±1.0$^{a}$§ | 12.5±0.4$^{c}$§ | 8.1±0.2$^{d}$§ | fermented |
| 1280 | 2-heptanol | 0.7±0.1$^{a}$* | 0.4±0.0$^{b}$* | 0.6±0.1$^{a}$* | 0.7±0.1$^{a}$* | 0.4±0.0$^{b}$* | 0.3±0.1$^{a}$§ | 2.2±0.3$^{b}$§ | 0.3±0.0$^{a}$§ | 0.2±0.0$^{a}$§ | 0.2±0.0$^{a}$§ | fruity, fresh lemon |
| 1460 | 2,6-dimethyl-4-heptanol | 2.2±0.3$^{a}$* | 0.3±0.0$^{b}$ | 4.0±0.2$^{c}$* | 1.7±0.2$^{a}$* | 4.3±0.5$^{c}$* | 9.3±1.1$^{a}$§ | nd | 14.5±1.7$^{b}$§ | 11.8±1.5$^{abd}$§ | 11.5±0.2$^{d}$§ | fermented yeasty |
| 1925 | phenethyl alcohol | 1.6±0.2$^{a}$* | 1.6±0.1$^{a}$* | 1.9±0.1$^{a}$* | 2.9±0.1$^{c}$* | 2.5±0.1$^{c}$ | 2.2±0.3$^{a}$§ | 0.2±0.0$^{b}$§ | 3.7±0.4$^{c}$§ | 5.0±0.2$^{d}$§ | 2.9±0.2$^{e}$ | floral rose |
| | **Acids** | | | | | | | | | | | |
| 1446 | acetic acid | 33.9±0.8$^{a}$* | 34.3±1.1$^{a}$ | 21.2±3.1$^{b}$ | 16.0±0.1$^{c}$ | 20.1±1.7$^{b}$* | 21.7±0.5$^{a}$§ | 31.9±3.6$^{b}$ | 23.2±3.5$^{a}$ | 16.7±1.1$^{c}$ | 23.3±2.9$^{a}$§ | acidic, pungent sour vinegar |
| 1474 | formic acid | 0.1±0.0$^{a}$* | nd | nd | nd | 0.5±0.1$^{b}$ | 1.6±0.1$^{a}$§ | 0.7±0.0$^{b}$ | nd | nd | 0.5±0.0$^{c}$ | acidic, pungent vinegar |
| 1480 | propanoic acid | 0.1±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.2±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.1±0.0$^{a}$ | acidic, pungent, dairy-like |
| 1580 | isobutanoic acid | 0.3±0.0$^{a}$ | 0.4±0.1$^{a}$ | 0.3±0.1$^{a}$ | 0.1±0.0$^{b}$ | 0.3±0.0$^{a}$ | 0.4±0.1$^{a}$ | 0.3±0.0$^{a}$ | 0.4±0.0$^{a}$ | 0.3±0.0$^{a}$ | 0.4±0.0$^{a}$ | acidic, dairy creamy |
| 1605 | butanoic acid | 1.3±0.1$^{a}$ | 0.9±0.0$^{b}$* | 0.8±0.0$^{b}$* | 0.9±0.1$^{b}$* | 1.1±0.0$^{a}$ | 1.5±0.1$^{ab}$ | 1.8±02$^{a}$§ | 1.3±0.1$^{bc}$§ | 1.3±0.1$^{bc}$§ | 1.0±0.1$^{c}$ | cheesy, dairy-like |
| 1684 | pentanoic acid | 1.3±0.1$^{a}$* | 1.1±0.1$^{a}$* | 1.0±0.1$^{a}$ | 0.3±0.0$^{b}$* | 1.0±0.0 $^{a}$* | 1.8±0.1$^{a}$§ | 0.4±0.0$^{b}$§ | 1.2±0.1$^{c}$ | 1.2±0.1$^{c}$§ | 2.0±0.1$^{a}$§ | cheesy, dairy milk cheesy |
| 1843 | hexanoic acid | 7.6±0.9$^{a}$ | 6.1±0.3$^{b}$* | 5.5±0.0$^{c}$* | 5.8±0.1$^{b}$* | 6.3±0.3$^{b}$ | 8.4±0.7$^{a}$ | 8.7±0.7$^{a}$§ | 6.7±0.6$^{bc}$§ | 7.8±0.8$^{ab}$§ | 6.7±0.1$^{c}$ | sweet cheese, yogurt-like aroma |
| 1978 | heptanoic acid | 0.3±0.0$^{a}$ | 0.2±0.0$^{a}$* | 0.3±0.0$^{a}$ | 0.1±0.0$^{a}$ | 0.3±0.0$^{a}$ | 0.3±0.0$^{a}$ | 0.5±0.0$^{b}$§ | 0.2±0.0$^{a}$ | 0.2±0.0$^{a}$ | 0.4±0.0$^{b}$ | cheesy sweat |
| 2057 | octanoic acid | 6.4±1.0$^{ab}$ | 6.6±0.1$^{a}$* | 6.7±0.6$^{a}$ | 5.5±0.3$^{b}$* | 8.0±1.2$^{a}$ | 8.0±1.0$^{ac}$ | 10.4±0.5$^{b}$§ | 7.1±0.4$^{a}$ | 7.9±0.2$^{ac}$§ | 8.0±0.2$^{c}$ | fatty, cheesy |
| 2159 | nonanoic acid | 0.3±0.0$^{a}$ | 0.3±0.0$^{a}$* | 0.9±0.1$^{b}$* | 0.1±0.0$^{c}$ | 0.4±0.0$^{a}$ | 0.2±0.0$^{a}$ | 0.5±0.0$^{b}$§ | 0.2±0.0$^{a}$§ | 0.1±0.0$^{a}$ | 0.3±0.0$^{a}$ | fatty, cheese, dairy |
| 2263 | decanoic acid | 3.3±0.5$^{a}$ | 2.6±0.3$^{a}$* | 1.5±0.2$^{b}$* | 2.1±0.0$^{c}$ | 3.4±0.3$^{a}$ | 4.0±0.4$^{a}$ | 5.5±0.8$^{b}$§ | 2.8±0.0$^{c}$§ | 1.8±0.2$^{d}$ | 3.2±0.2$^{e}$ | fatty, unplesant rancid |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2340 | 9-decenoic acid | 0.3±0.0$^{a}$ | 0.2±0.0$^{a*}$ | 0.3±0.0$^{a}$ | 0.2±0.0$^{a}$ | 0.4±0.1$^{a}$ | 0.5±0.2$^{ac}$ | 0.6±0.1$^{a\S}$ | 0.2±0.0$^{b}$ | 0.2±0.0$^{b}$ | 0.3±0.0$^{c}$ | fatty, waxy |
| 2430 | benzoic acid | 3.4±0.3$^{a*}$ | 3.4±0.3$^{a}$ | 3.6±0.2$^{a*}$ | 3.0±0.1$^{a*}$ | 4.3±0.1$^{b*}$ | 2.6±0.3$^{ab\S}$ | 3.0±0.1$^{a}$ | 2.5±0.2$^{b\S}$ | 2.4±0.3$^{b\S}$ | 3.0±0.4$^{ab\S}$ | fruity, alcholic |
| 2470 | dodecanoic acid | 0.8±0.1$^{a}$ | 0.5±0.0$^{b*}$ | 0.8±0.1$^{a*}$ | 0.3±0.0$^{b}$ | 0.9±0.1$^{a}$ | 1.0±0.1$^{a}$ | 1.1±0.1$^{a\S}$ | 0.4±0.0$^{b\S}$ | 0.4±0.0$^{b}$ | 0.7±0.1$^{c}$ | fatty, coconut |

RI = Retention Index, identification by comparison with RI database; nd = not detected. Results are expressed as RAP= Relative Peak Area (Area Peak Compound/Area Peak Internal Standard) 100.

* Based on flavornet (www.flavornet.org) and pherobase (www.pherobase.com) online databases. For traditional kefir or backslopped kefir, lowercase letters (a, b, c, d, e) indicate significant differences ($p < 0.05$) in relative volatile compounds within the different culture types (A, B, C, D, E). Symbols (*,$^{\S}$) indicate significant differences ($p < 0.05$) in relative volatile compounds among traditional kefir and the respective backslopped kefir.

Graphical abstract



**Highlights**

- The microbial dynamics and volatilome profile in kefir production have been studied
- A microbial shift was observed during the production of backslopped kefir
- Backslopped kefir samples showed higher pH, protein, lactose and ash content
- Backslopped kefir was correlated with cheesy, buttery, floral and fermented odors
- This is the first report on volatilome profile of the backslopped kefir

**Credit author statement**

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