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ARTICLE

Volatilome changes during probiotic fermentation of combined plant-based drinks

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Plant-based drinks as a substitute for animal milk consumption are crucial products in the food industry. Soy and rice drinks are the most successful milk substitutes but are low in fiber and protein contents, respectively, whilst rich in sugars. Generally, an improvement is foreseen; thus, apart from supplements addition, a natural occurring strategy is functionalizing the drinks by beneficial bacteria fermentation. The aim of this work is to develop novel plant-based drinks assessing different mixtures of soy and rice milks fermented with single or multi-strains probiotics (*Lactobacillus fermentum*, *L. plantarum, L. helveticus*, *Bifidobacterium bifidum,* and *B. longum*). The drinks were characterized to study bacterial performances, by means of culture-dependent and -independent techniques, and their volatilome, by means of solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) analysis. Through multivariate analysis, these features were investigated and correlated to define accurate descriptors of the produced functional drinks. The results showed that combined drinks and multi-strains fermentation generated higher-value products. For example, blended drinks in comparison to single ones had lower amount of toxic 2-acetyl-3,5-dimethylfuran and higher abundances of desirable compounds as 2-butanone, 3-hydroxy and butanoic acid. Multivariate analysis of volatile metabolites and physiological parameters could offer a novel approach to assess the quality of functional plant-based drinks and result in a decisional tool for industrial applications.

1. Introduction

Milk is one of the most consumed foods, however, cow's milk allergy is about 2% - 3% in early childhood[1] and lactose intolerance, a deficiency of lactase which results in gastrointestinal disorders, affects 65% of the world's population[2,3]. In addition, there is a growing health awareness among consumers, who are increasingly directed towards alternative dairy products[4]. To meet consumer demands, alternative drinks to cow's milk have been developed, produced from matrices such as fruit, cereals, and soybeans. Soy-based drinks are one of the most common, known for the ability of soy to lower blood pressure and potentially prevent chronic and degenerative diseases, thanks to the presence of antioxidant compounds such as isoflavones[5,6]. Soy also boasts a high protein content, vitamins such as vitamin B1, vitamin B9, vitamin B2 and vitamin K, and minerals such as phosphorus and magnesium[7]. Moreover, soy-based drinks contain few saturated fatty acids and is completely free of cholesterol or lactose[8]. Rice-based drink is also cholesterol-free but, unlike soy drink, it also completely lacks unsaturated fats and allergens[9]. Another advantageous characteristic of rice-based drink is its high content of selenium and magnesium, which hinder the development of bacteria and viruses[9]. Otherwise, soy and rice drinks are low in fiber and proteins contents, respectively, whilst rich in sugars. Generally, an improvement is necessary, and apart from supplements addition, a simple and natural occurring strategy could be that of blending and functionalizing the drinks with beneficial bacteria fermentation. Different cereals or legumes have different and complex biochemical compositions and when mixed modify the sensorial and biological characteristics of the final product. Mixing can affect certain compounds and improve the diversity and accessibility of fermentable substrates, influencing growth and metabolism of microorganisms. On the other hand, fermentation by lactic acid bacteria can increase the functional and sensorial worth of the final product, in particular the use of multi-strains bacterial cultures can exploit the fermentation outputs. Fermentation of plant-based drinks is mainly conducted with lactobacilli, that are able to exalt the bio accessibility of many different bioactive compounds and increase their yields[10]. These bacteria are able to resist and grow in difficult environment, such as that of plant-base drinks characterized by extreme acidity and the presence of inhibitory factors[11,12]. In particular, lactobacilli and bifidobacteria harbor different alpha-glucosidases that make them ideal to ferment plant-based stuff[13]. Moreover, many strains of these groups are beneficial, and others are probiotics, conferring health benefits to the host[14]. In fermented plant-based products, the metabolic outcomes derived from the process are scarcely studied and the distribution of metabolites during fermentation should be monitored, because the success of a new non-dairy drink is undoubtedly linked to its organoleptic characteristics, which will determine consumer's acceptance. For this purpose, the study of

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the volatilome of the products, which represent the total volatile organic compounds (VOCs) including sensorial and bio-active types, fits perfectly. Metabolomics has been already applied to study fermented foods, such as dairy, bakery products, wines, plant-based drinks[15-21]. The aim of this study is to prepare and characterize a new functional plant-based drink fermented with beneficial lactobacilli and bifidobacteria, focusing on the comparison among single or blended matrices fermented singularly or by multi-strain cultures. To achieve the main objective, we employed an omic approach exploring the product's volatilome and its correlations with microbiological features of fermentation.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

All bacterial strains tested belong to the microbial collection of DISTAL (Dept. of Agricultural and Food Sciences), University of Bologna (Bologna, Italy) and have been previously isolated from plant-based products and extensively studied[17,18,20,22,23]. Bacteria were obtained from 30% (*v/v*) glycerol stocks stored at -80 °C and were propagated in de Man Rugosa Sharpe (MRS) (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) broth (Dextrose 20%; Peptone 10%; Beef Extract 8%; Sodium Acetate 5%; Yeast Extract 4%; Ammonium Citrate 2%; Dipotassium Phosphate 2%; Polysorbate80 1%; Magnesium Sulfate 0.2%; Manganese Sulfate 0.05%) containing L-cysteine 0.05% (*v/v*) (Sigma, St. Louis, MO, USA), at 37 °C in microaerophilic conditions, applying jars with oxygen catalyst (Oxoid, Thermo Fisher Scientific, USA) for 48 h.

2.2. Drinks Preparation

The plant-based drinks used in this research are commercial organic certified drinks. Soy drink (S) (Alinor, Ripalta Cremasca, Italy), as stated by label, contains water, soy 8% (*v/v*), and sea salt. Rice drink (R) (Alinor, Italy), as stated by label, contains water, rice 17% (*v/v*), sunflower oil cold-pressed, and sea salt. All commercial products used in this study were UHT (Ultra-High Temperature) treated. Before fermentation, the drinks were prepared as single matrix, such as soy (S) and rice (R) or in blends. The blends were prepared aseptically as follow: 25% (*v/v*) of soy drink and 75% (*v/v*) of rice drink (SR25); 50% (*v/v*) of soy drink and 50% (*v/v*) of rice drink (SR50); 75% (*v/v*) of soy drink and 25% (*v/v*) of rice drink (SR75).

2.3. Fermentations

The vegetable drink samples were fermented independently by *Lactobacillus helveticus* (lh) CNBL, *Lactobacillus rhamnosus* C243 (lr), *Bifidobacterium bifidum* B700795 (bb), and *Bifidobacterium longum* Bb12 (bl), and by two bacterial mixes m1 and m2. The former mix (m1) contained equal proportion of lh, lr and bb, while the latter (m2) contained equal proportion of lh, lr and bl. Cell load of inoculated bacteria was standardized at Log 6 CFU/mL (Colony Forming Unit/mL). Fermentation of the beverages was conducted in 50 mL of final volume and incubated for 24 h at 37 °C in jars with anaerobiosis catalyst (Thermo Scientific, USA). Not inoculated drinks were used as controls. Two biological replicates of each formulation were performed in different periods. At time zero, after 6 h, and at the end of fermentation (24 h), bacterial growth and pH were monitored, while volatile organic compounds (VOCs) were analyzed at the beginning and at the end of the experiment. Prior to conduct fermentation, bacterial propagation was achieved at least for two following times. Bacterial load of the inocula was obtained by spectrophotometry means and measured afterward by bacterial plating. Bacterial cells were centrifugated and resuspend two times in sterile water before addition to the experimental drinks for the fermentation. Sample codes description can be found in Table S1.

2.4. Bacterial Quantification

Bacterial quantification was obtained by both culture-dependent and culture-independent protocols. The culture-dependent protocol was achieved by plating on selective MRS agar (Oxoid, Thermo Fisher Scientific, USA) supplemented with 0.05 g/L L-Cysteine (Sigma, USA) serial dilutions of the samples made in physiological solution (0.9% NaCl) and incubating for 24 h at 37 °C in microaerophilic conditions, using jars with oxygen catalyst (Thermo Fisher Scientific, USA). Plate count method was performed in duplicates from each independent experiment. Culture-independent quantifications were obtained by qPCR with the SYBR Green I chemistry, applying genus-specific primers as Lac1 for *Lactobacillus* spp. (forward:5'-GCAGCAGTAGGGAATCTTCCA-3' and reverse: 5'-GCATTYCACCGCTACACATG-3')[24] and RecA for *Bifidobacterium* spp. (forward: 5'-CGTYTCBCAGCCGGAYAAC-3' and reverse: 5'-CCARVGCRCCGGTCATC-3')[25]. Templates for qPCR to generate standard curves were amplified by PCR using a ProFlex PCR System apparatus (Thermo Fisher Scientific, USA) with SuperFi Platinum Taq (Thermo Fisher Scientific, USA). Amplicons were purified with a commercial DNA purification system (GeneJet PCR purification kit, Thermo Fisher Scientific, USA). qPCR was performed with a RotorGene 6000 (Qiagen, Hilden, Germany) and the RotorGene Q Series Software 2.3.1 Release (Qiagen, Germany). DNA extraction, standards preparations, PCR, and qPCR reactions were performed according to previously published protocols[17-19]. qPCR analysis was performed in triplicates from each independent experiment.

2.5. pH

pH was determined with a pH meter (Crison, Alella, Spain) at 20 °C, appropriately calibrated with three standard buffer solutions at pH 9.21, pH 4.00, and pH 2.00. The pH measurements were conducted at three different time points to monitor the fermentation, and values were expressed as the means of triplicates from each independent experiment.

2.6. Solid-Phase Microextraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS)

Evaluation of volatile organic compounds (VOCs) was carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV), equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The SPME-GC-MS protocol and the identification of volatile compounds that was employed were previously published[17,18,23]. Before each head space sampling the fiber was exposed to the GC inlet for 10 min for thermal desorption at 250 °C in a blank sample. The samples were then equilibrated for 10 min at 50 °C. The SPME fiber was exposed to each sample for 40 min and finally the fiber was inserted into the injection port of the GC for a 10 min sample desorption. The temperature program was: 50 °C for 0 min, then ramping at 1.5 °C/min to 65 °C and at 3.5 °C/min to 220 °C, which was maintained for 20 min. Injector, interface, and ion source temperatures were 250 °C, 250 °C, and

230 °C, respectively. Injections were carried out in splitless mode and helium (3 mL/min) was used as carrier gas. Identification of molecules was carried out by comparing their retention times with those of pure compounds (Sigma, USA) and confirmed by searching mass spectra in the available databases (NIST version 2005 and Wiley version 2016) and literature. The main fermentation metabolites (ethyl alcohol, acetic acid, lactic acid, and 2-butanone-3-hydroxy) were quantified in mg/kg using an internal standard, while all other VOCs were relatively quantified in percentage as described previously[17,23].

2.7. Statistical Analyses

All statistical analyses were performed using TIBCO Statistica 8.0 (Tibco Inc., Palo Alto, CA, USA). Normality was checked with the Shapiro–Wilks test and homoscedasticity was evaluated with the Levene's test [26]. Differences between all samples were evaluated with Multivariate Analysis of Variance (MANOVA), while Principal Component Analysis (PCA), K-mean clustering, Spearman Rank Correlations, and Two-way joining heatmap were used to study the relationship between the variables. For post hoc test, a Tukey's test was employed. For multivariate analysis, the data were normalized using the mean centering method. All results are expressed as mean values obtained at least from duplicate batches in two independent experiments.

3. Results

3.1. Bacterial Quantification and pH Values during the Process

Quantification of bacterial species obtained by plate count and qPCR are shown in Table S2. Results are expressed as Log_{10} cell/ml and represent the mean value of the two methods since they give similar results when are applied to quantify the evolution of a known and standardized inoculum in a simple microbial-free food matrix made in a research laboratory[23,24] . In combined drinks, the bacterial load resulted higher than in single drinks, particularly at the early time point (6 h) when the difference was about 0.96 ± 0.68 Log_{10} cell/ml ($P < 0.05$). The maximum bacterial load was achieved by SR75 fermented with m1, accounting for 10.37 ± 0.35 Log_{10} cell/ml and a delta increment from baseline to endpoint of 3.24 ± 0.05 Log_{10} cell/ml. Considering pH (Table S3), no significative difference were found between blend and single drinks ($P > 0.05$) *L. helveticus* (lh) was the starter that almost in every substrate had the strongest acidification. In details, at the early time point of fermentation lh acidification was stronger in blend drinks, reaching the top in SR25 (pH 4.28 ± 0.02), instead, at the late time point reached the top in single rice drinks (3.01 ± 0.06).

3.2. Analysis of the Volatilome

Volatilome analysis identified more than 200 molecules and 76 resulted normally distributed ($P < 0.05$) and then were relatively quantified. For a landscape description of the volatilome a dataset normalized with the mean centering method including all cases and variables was proposed to generate a quantification heatmap (Figure S1) and ANOVA of sums of molecules comparing samples prior and after fermentation (Figure 1). To investigate in detail the effects of cases on dependent variables, a multivariate approach was conducted independently on 4 different normalized datasets regarding: i) alcohols (without ethyl alcohol); ii) aldehydes; iii) ketones; iv) organic acids. These datasets were computed for Principal Component Analysis (PCA) and K-means Clustering Analysis to describe samples by molecules (Fig. 2-5); MANOVA with categorical predictors to weight the contribution of bacterial strains or drink formulations in VOCs generation (Fig S2); Spearman Rank Analysis to define correlations between VOCs generation and fermentation parameters (bacterial growth, pH, main fermentation metabolites) (Fig. 6). The main fermentation metabolites (ethyl alcohol, acetic acid, lactic acid, and 2-butanone-3-hydroxy) (Table 1) were quantified in mg/kg using an internal standard as described previously[18,19,23].

3.2.1. Characterization of VOCs profiles. Eighty-eight VOCs, including 19 alcohols, 12 aldehydes, 16 ketones, 12 organic acids, 12 alkanes, 10 amines, and 7 alkenes, were relatively quantified from drinks prior and after fermentation. A higher quantity and a wider speciation of compounds was identified in fermented samples. Overall, single drinks and not fermented drinks were characterized by a greater presence of aldehydes and ketones, on the contrary the combined drinks showed a greater presence of alcohols and organic acids. The heatmap of relative quantification of VOCs in the different samples is reported in supplementary material (Figure S1).

3.2.2. Effect of Fermentation. In all sample fermentation caused a significant high increase in alcohols and organic acids ($P < 0.05$), a significant minor increase in aldehydes and ketones ($P < 0.05$), while for alkanes, amines and alkenes no significant differences were found over the process ($P > 0.05$). Generally, after fermentation samples had 1.8-, 13.4-, 3.0-, and 12.3-time more aldehydes, alcohols, ketones, and organic acids. After fermentation, the SR50 series was that accounting for largest yields of almost every chemical class of compounds. When SR50 series was compared to the not fermented drinks series, it had 17.2- and 16.1-times the concentrations of alcohols and organic acids, respectively (Figure 1). Combined samples in comparison to single drinks had averagely a higher amount of every chemical class, in particular SR50 had the double quantity of aldehydes than single soy drink.

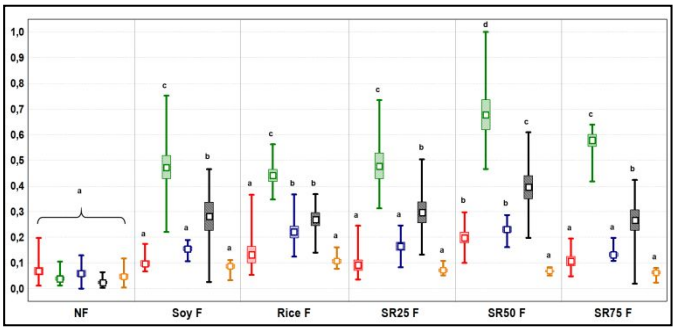

Figure 1. Relative quantification of total volatile organic compounds (VOCs) divided by chemical classes prior and after fermentation. Different letters indicate different significance values by Tukey's HSD (honestly significant difference) test ($P < 0.05$). Sample abbreviations: NF = not fermented samples; F = fermented samples. SR25 = Blend with 25% (v/v) soy drink and 75% (v/v) rice drink; SR50 = Blend with 50% (v/v) soy drink and 50% (v/v) rice drink; SR75 = Blend with 75% (v/v) soy drink and 25% (v/v) rice drink. Square = mean; box = mean ± Standard Deviation (SD); whiskers = max & min. Red plots = aldehydes; green plots = alcohols; blue plots = ketones; black plots = organic acids; yellow plots = others (alkanes, amines, alkenes).

3.2.3. Quantifications of the Main Fermentation Metabolites.

Quantification of main fermentation metabolites (mg/kg of fermented matrix) is reported in Table 1. For every fermented drink, higher proportion of soy (SR75) generates higher concentration of ethanol. 2-butanone,3-hydroxy in combined drinks scored the highest concentration compared to single drinks. In particular, fermentations conducted by *B. bifidum* 700795 produced in the SR50 series 1.87-fold more 2-butanone,3-hydroxy than single soy drink and 2.31-fold more than single rice drink ($P < 0.05$). Also in the production of acetic acid the combined drinks performed better than single drinks, in particular it reached the top concentrations in SR75 series fermented by *B. bifidum* 700795 (bb) and by m1, which both produced 5-fold more ($P < 0.05$) of acetic acid compared to single soy drink. Finally, combined drinks also produced more lactic acid than the single drinks. In particular, the SR25 series fermented with *L. rhamnosus* C243 was the most performing, producing 3.88-fold more than single rice drink ($P < 0.05$).

Table 1. Means values in mg/Kg of main metabolites from bacterial fermentation of drinks.

Sample	ethyl alcohol	2-butanone,3-hydroxy	acetic acid	lactic acid
S	n.d.	n.d.	n.d.	n.d.
R	n.d.	n.d.	n.d.	n.d.
SR25	n.d.	n.d.	n.d.	n.d.
SR50	n.d.	n.d.	n.d.	n.d.
SR75	n.d.	n.d.	n.d.	n.d.
Slh	12.16 ± 0.02^{c}	tr.	tr.	0.24 ± 0.23^{a}
Slr	25.20 ± 0.02^{d}	0.45 ± 0.04^{b}	1.55 ± 0.04^{b}	0.32 ± 0.04^{a}
Sbb	19.46 ± 0.04^{d}	0.48 ± 0.03^{b}	1.65 ± 0.06^{b}	0.14 ± 0.03^{a}
Sm1	19.46 ± 0.04^{d}	0.48 ± 0.03^{b}	1.65 ± 0.13^{b}	0.14 ± 0.04^{a}
Sbl	13.17 ± 0.09^{c}	0.40 ± 0.04^{b}	0.50 ± 0.08^{a}	0.15 ± 0.03^{a}
Sm2	13.28 ± 0.02^{c}	0.27 ± 0.04^{a}	1.53 ± 0.23^{b}	0.12 ± 0.06^{a}
Rlh	5.63 ± 0.05^{b}	0.52 ± 0.04^{b}	1.45 ± 0.13^{b}	0.17 ± 0.08^{a}
Rlr	12.96 ± 0.45^{c}	0.41 ± 0.01^{b}	1.23 ± 0.12^{b}	0.43 ± 0.08^{b}
Rbb	1.45 ± 0.89^{a}	0.39 ± 0.08^{b}	0.44 ± 0.07^{a}	0.13 ± 0.07^{a}
Rm1	15.70 ± 0.37^{c}	0.31 ± 0.01^{a}	1.56 ± 0.25^{b}	0.018 ± 0.06^{a}
Rm1	15.70 ± 0.37^{c}	0.31 ± 0.01^{a}	1.56 ± 0.25^{b}	tr.
Rbl	1.95 ± 0.18^{a}	0.44 ± 0.04^{b}	0.37 ± 0.15^{a}	tr.
Rm2	6.80 ± 0.40^{b}	0.40 ± 0.03^{b}	1.53 ± 0.26^{b}	tr.
SR25lh	5.63 ± 0.05^{b}	0.52 ± 0.04^{b}	7.23 ± 0.15^{c}	0.87 ± 0.09^{c}
SR25lr	12.96 ± 0.45^{c}	0.41 ± 0.01^{b}	6.15 ± 0.11^{c}	1.67 ± 0.44^{c}
SR25bb	0.45 ± 0.52^{a}	0.39 ± 0.07^{b}	2.22 ± 0.08^{b}	0.56 ± 0.04^{b}
SR25m1	15.70 ± 0.37^{c}	0.31 ± 0.03^{a}	7.78 ± 0.53^{c}	0.91 ± 0.23^{c}
SR25bl	0.57 ± 0.34^{a}	0.44 ± 0.18^{b}	1.85 ± 0.06^{b}	0.44 ± 0.06^{b}
SR25m2	6.80 ± 0.40^{b}	0.40 ± 0.01^{b}	7.64 ± 0.84^{c}	0.41 ± 0.03^{b}
SR50lh	11.67 ± 0.45^{c}	0.51 ± 0.03^{b}	7.84 ± 0.25^{c}	0.96 ± 0.38^{c}
SR50lr	20.25 ± 0.47^{d}	0.59 ± 0.03^{c}	7.14 ± 0.48^{c}	1.24 ± 0.32^{c}
SR50bb	0.52 ± 0.05^{a}	0.90 ± 0.02^{c}	1.87 ± 0.07^{b}	0.54 ± 0.02^{b}
SR50m1	4.94 ± 0.46^{b}	0.75 ± 0.03^{c}	7.65 ± 0.44^{c}	0.70 ± 0.22^{b}
SR50bl	0.22 ± 0.29^{a}	0.71 ± 0.03^{c}	2.55 ± 0.11^{b}	0.56 ± 0.17^{b}
SR50m2	12.03 ± 0.13^{c}	0.48 ± 0.09^{b}	8.06 ± 0.61^{c}	0.80 ± 0.15^{c}
SR75lh	12.16 ± 0.02^{c}	0.36 ± 0.21^{b}	0.22 ± 0.06^{a}	0.66 ± 0.19^{b}
SR75lr	25.20 ± 0.02^{d}	0.45 ± 0.04^{b}	7.77 ± 0.02^{c}	1.08 ± 0.20^{c}
SR75bb	9.46 ± 0.03^{b}	0.48 ± 0.03^{b}	8.24 ± 0.65^{c}	0.71 ± 0.17^{b}
SR75m1	19.46 ± 0.04^{d}	0.48 ± 0.04^{b}	8.24 ± 0.22^{c}	0.72 ± 0.17^{b}
SR75bl	13.17 ± 0.09^{c}	0.40 ± 0.06^{b}	2.50 ± 0.01^{b}	0.70 ± 0.17^{b}
SR75m2	13.28 ± 0.02^{c}	0.27 ± 0.03^{a}	7.64 ± 0.28^{c}	0.45 ± 0.03^{b}

Values are means of two replicates and two different batches. * traces = values < 0.1 mg/kg; † n.d. = not determined. Different letters in the same column indicate significant differences (at least $P < 0.05$). For samples abbreviations see Table S1.

3.2.4. Multivariate Analysis of VOCs Organized by Different Chemical Classes

Alcohols. To better evidence differences in the alcohol class, normalization of the dataset and statistical analysis were performed after exclusion of ethanol, whose weight was overwhelming. 19 different alcohols over 72 cases were processed by PCA that grouped samples in different directions on the plane where K-means analysis identified four clusters (Figure 2). Three clusters were made by fermented and one by NF samples. Cluster 1 included drink combinations fermented by single inocula. This cluster was described by 15 compounds, among that nonanol,5-ethyl, 2,4,4,trimethyl-1-pentanol, and 2-dodecanol were more abundant than in other clusters. Cluster 2 contained mainly combined drinks fermented with bifidobacteria or both the mixes (12 out of 16 combined drinks fermented by the mixes). It was described by all variables, but one, and it was addressed by higher concentrations of 2-decanol, 1-penatol, 1-hexanol, heptanol, 3-tridecanol, and 2-octen-1-ol (E). Cluster 3 was positioned oppositely to the previous and had members of almost all single drinks

fermented samples, regardless from the inoculum used. It was described by lower speciation made by just 8 alcohols and by a typical signature made by pentanol-5-amino and 2-hexadecanol. Lastly, cluster 4 was set on quadrant I and contained NF samples described by traces amounts of 13 alcohols. Results from MANOVA categorized for the matrix ($P < 0.001$) (Figure S2A) indicated that combined drinks generally accounted for a higher percentage of every alcohols in respect to the single drinks. In particular SR50 were the sole responsible for 2-dodecanol production, while the SR75 series produced almost the 80% of 3-tridecanol. MANOVA categorized for the strains ($P < 0.01$) (Figure S2B) showed that, *L. rhamnosus* C243 characterized the 45% of 2-octen-1-ol (E) production, *B. longum* Bb12 the 48% of 2-hexadecanol, bacterial mix1 the 42% of heptanol, and bacterial mix2 the 40% of 1-nonanol. Instead, *L. helveticus* CBNL and *B. bifidum* 700795 did not characterize in particular the production of any alcohols.

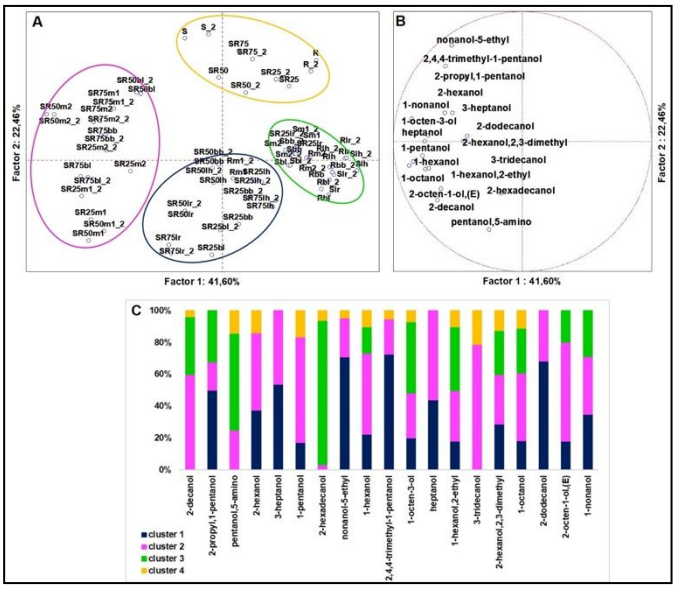

Figure 2. (A) Principal component analysis (PCA) of cases and variables on alcohols ($P < 0.05$); (B) K-means clustering analysis (at least $P < 0.05$).

Aldehydes. 12 different aldehydes were spread on PCA plane, where K-means identified four clusters among the 72 cases (Figure 3). Cluster 1 included only NF drinks, in particular 6 out of 10 totals, and had maximum loads of furfural. Cluster 2 was positioned on the upper left quadrant and contained mainly combined drinks fermented with bifidobacteria and with both the mixes (12 of the 16 combined drinks fermented by the mixes). This cluster was described by 7 compounds and it was characterized by an higher concentration of 2-heptenal,(Z), hexanal, heptanal and octanal. Cluster 3 was in the opposite position from the previous and contained all the fermented single soy drinks. It was described by 11 alcohols with the highest concentration of butanal,3-methyl. Cluster 4 mainly included combined and single drinks fermented by lactobacilli and it was described by traces amounts of 9 aldehydes whit top values of decanal. Results from MANOVA categorized for the matrix ($P < 0.05$) (Figure S2C) showed no significant differences for the production of aldehydes in single or combined drinks, except for 2,4-heptadienal,(E,E) which is produced exclusively by the fermentation of combined drink. MANOVA categorized for the strains ($P < 0.01$) (Figure S2D) showed that *L. helveticus* CBNL characterized about 70% of butanal,3-methyl production, *L. rhamnosus* C243 about 60% of 2-butenal,3-methyl production, and *B. longum* Bb12 about 40% of 2-heptenal,(Z) production. *B. bifidum* 700795 and both the bacterial mixes showed no significant contributions in the production of any aldehydes.

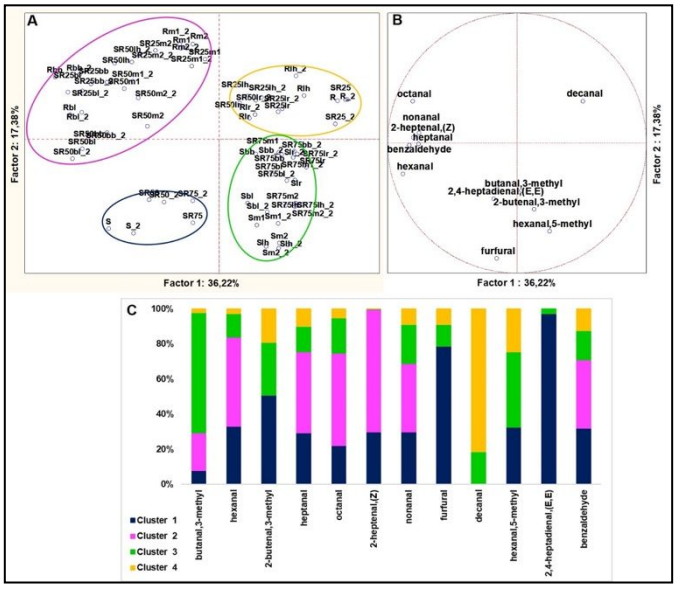

Figure 3. (A) PCA of cases and variables on aldehydes ($P < 0.05$); (B) K-means clustering analysis (at least $P < 0.05$).

Ketones. 13 different ketones were spread on PCA plane where K-means identified five clusters among the 72 cases (Figure 4). Cluster 1 included principally drink combinations and was described by 8 compounds, whit 3-nonanone,2-methyl as more abundant than in other clusters. Cluster 2 contained mainly NF samples and It was distinguished by higher concentrations of 2-pentanone, 6-dodecanone and 1-propanone,1-cyclohexyl. Cluster 3 included almost all fermented single soy drinks described by a large speciation (13 ketones), but low abundances and no exclusive signature. Cluster 4 contained every fermented SR75 cases, except those with lh. It was characterized by a large speciation with top values of 11-dodecen-2-one, 7-pentadecanone and 2-nonanone. Cluster 5 included 8 of the 12 blends fermented by the two bacterial mixes. This cluster was described by 8 ketones with a higher concentration of 4-heptanone,2,6-dimethyl and 2-butanone. Results from MANOVA categorized for the matrix ($P < 0.05$) (Figure S2E) indicated that combined drinks were responsible for about 50% of 3-penten-2-one,4-methyl, 2-butanone,3-hydroxy, and 4-heptanone,2,6-dimethyl production. MANOVA categorized for the strains ($P < 0.001$) (Figure S2F) showed that *B. longum* Bb12 characterized about 40% of 2-butanone production while *L. helveticus* CBNL about 40% of 3-nonanone,2-methyl. Bacterial mixes mainly contributed to the production of 2-nonanone, in particular mix1 produced almost its 40%.

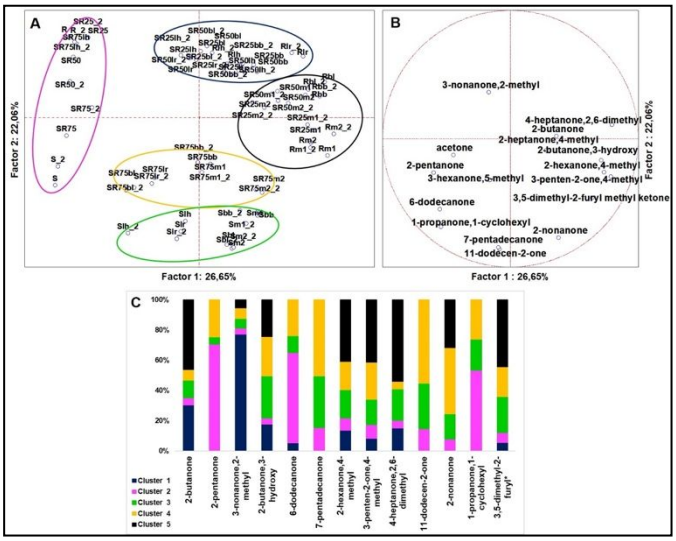

Figure 4. (A) PCA of cases and variables on ketones (P < 0.05); (B) K-means clustering analysis (at least P < 0.05).

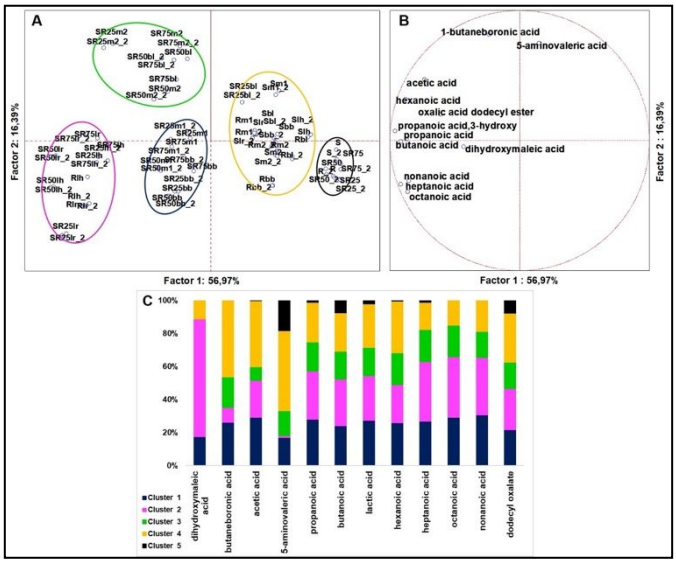

Figure 5. (A) PCA of cases and variables on organic acids (P < 0.05); (B) K-means clustering analysis (P < 0.05). For samples' abbreviations see Table S1 or paragraphs.

Organic Acids. 12 different ketones were spread on PCA plane where K-means identified five clusters among the 72 cases (Figure 5). Four clusters were made by fermented samples and one by NF samples. Cluster 1 included all the combined drinks fermented with *B. bifidum* 700795 and with m1. This cluster was addressed by higher concentrations of acetic, propanoic, octanoic, and nonanoic acids. Cluster 2 contained all the combined and the single rice drinks fermented by lactobacilli. It was described by all variables with dihydroxymaleic acid as the most abundant over the dataset. Cluster 3 was made of all the combined drinks fermented by m2 and the majority of those fermented by *B. longum* Bb12. This cluster was described by all variables, except one, but without a typical signature. Cluster 4 contained almost all single fermented drinks (20 out of 24) and was described by higher abundances than in other clusters of butaneboronic acid and 5-aminovaleric acid. At last, Cluster 5 included only NF drinks was described just by traces amounts of some organic acids. Results from MANOVA categorized for the matrix ($P < 0.05$) (Figure S2G) indicated that combined drinks generally accounted for a higher percentage of organic acids in respect to the single drinks. Combined drinks were the main producers of heptanoic, octanoic (around 70% for each) and nonanoic (around 80%) acids. In particular, SR50 series was the main producer of dihydroxymaleic acid (around 50%) while SR75 of nonanoic acid (around 40%). MANOVA categorized for the strains ($P < 0.01$) (Figure S2H) showed that dihydroxymaleic acid was produced only by *L. rhamnosus* C243 (about 80%) and by m1. Moreover, *L. rhamnosus* C243 characterized the production of almost every organic acid while *B. bifidum* 700795 and *B. longum* Bb12 did not characterize any more than other inocula.

3.3. Correlations between VOCs and fermentation parameters

With the use of Spearman rank analysis, correlations between variables related to ecological features (bacterial growth, pH decrease, and main fermentation metabolites) and variables related to abundances of VOCs, on fermented cases (n = 60) were found (Figure 6). Considering acidification and bacterial growth, their delta values were obtained in respect to the endpoint. Significant correlations ($P < 0.05$) indicated that dihydroxymaleic, heptanoic, and octanoic acids were positively correlated to the acidification, while a negative but significant correlation ($P < 0.05$) was that related to 1-hexanol. Heptanol in our samples was significantly correlated to acetate ($P < 0.05$), as a result of fermentation. Otherwise, the correlation with bacterial growth and lactate was positive, but not significant ($P > 0.05$). In fact, heptanol is a typical alcohol of plant-based drinks, thus is intrinsic of the matrix. 1-Octen-3-ol in our samples was described by a positive and significative ($P < 0.05$) correlation with some fermentation products, such as acetate and lactate, and with the bacterial growth. 2-heptanone,4-methyl and 2-butanone were positively correlated with acidification but negatively correlated with the bacterial growth ($P < 0.05$). This meant that these two ketones served as substrates for microbial fermentation (acidification) more than for their growth (no significant correlation with this latter).

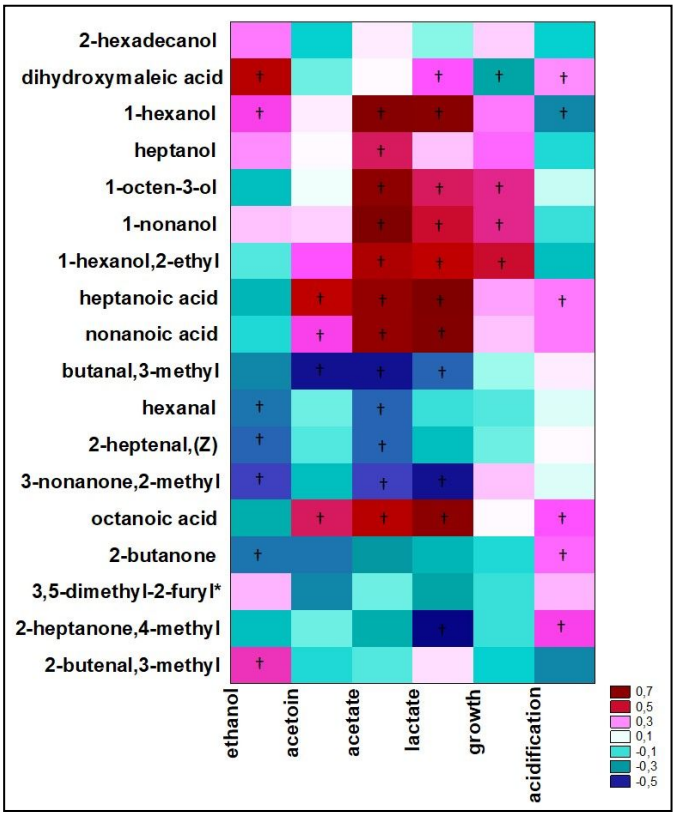

Figure 6. Spearman Rank Correlations Analysis between generation of VOCs and fermentation parameters at the end point of experiments. * 3,5-dimethyl-2-furyl methyl ketone; †Significant correlations ($P < 0.05$).

4. Discussion

4.1. Bacterial Growth and Major Fermentation Metabolites

Bacterial loads have been shown to be higher in blends compared to single drinks, probably due to the richer composition of the substrate, which is able to satisfy the complex nutritional needs of lactic bacteria. In our results, in fact, the maximum bacterial load was reached by SR75 fermented by m1. Another interesting concept highlighted by the present study is the use of mixed cultures to ferment plant-based drinks. In this type of bacterial culture, the two different genera mutually stimulate growth, the production of acids and VOCs[27]. A synergistic effect on growth for soybean fermentation was also observed, probably related to the different glycolytic activities of the strains involved[27]. pH values were technologically better in the blends in respect to the single drinks, showing a stronger acidification after 6 h and a milder at the endpoint. This issue is biologically and technologically desirable, because a fast acidification permits to counteract spoilage microbes to grow, while a reduced acidification at the late time point indicates a buffering capacity of the substrate that led to a product not too sour for consumers[17].

4.2. Multivariate Analysis of VOCs Sorted by Chemical Class

4.2.1. Alcohols. Alcohols are essential compounds of any microbial fermentation. Their contribution to food texture, microbial stability, and aroma is broad. Some are foreseen, as primary alcohols to counteract spoilage[28], others are forsaken as furfuryl alcohol for its acute toxicity[29]. Among minor alcohols, there was a clear distinction between those that characterized for the single drinks and those for the blends. In fact, speciation and abundances were larger and higher in combined drinks. In fact, the single drinks were described by 11 out 19 alcohols, while the blend drinks were described by every alcohol, except 2-hexadecanol. Another distinction is that the blends containing higher proportion of soy (SR75) were different from that with higher rice percentage (SR25). The former were plenty of 1-pentanol, 3-tridecanol, and 2-octen-1-ol (E), while the latter had more nonanol,5-ethyl, and 2,4,4,trimethyl-1-pentanol. Of note, all the blends were exclusively described by two heptanol isomers, that were not detected in single drinks. Heptanol, and 2-octen-1-ol (E) are associated to hemp drinks fermented with probiotics[17]. Heptanol is a typical alcohol of plant-based drinks, characterized by a typical olfactory issue described as musty, pungent, leafy, green[17]. 2-octen-1-ol (E) derives from linoleic acid oxidation, and it has antimicrobial activity against spoilage and food-borne pathogens[26]. 3-tridecanol is a long chain fatty alcohol that has a particular scent defined as musty and it is used in cosmetics and food industries as an emollient or masking agent[31]. 2-Octen-1-ol (E) is a common volatile compound reported to be a product derived from the oxidation of linoleic acid, which can be found in plants and fungi and characterized by antimicrobial activity[30]. Besides, it has attributes of antimicrobial activity versus cariogenic *Streptococcus mutans*[32], food-borne opportunistic *Acinetobacter calcoaceticus*[28], and pathogenic *Salmonella gallinarum*[33]. Our data evidenced positive correlations of heptanol with bacterial growth and fermentation metabolites in particular significantly with acetate increase. Likewise, octenol was positively correlated with bacterial growth, acidification, and acetate production, demonstrating not to be effective against beneficial microbes despite its antimicrobial nature.

4.2.2. Aldehydes. Aldehydes production in fermented food is a result of microbial fermentation and lipid oxidation[34]. For instance, aliphatic linear C10–C18 aldehydes are potent odoriferous components in perfumes[35]. Many aldehydes are required because they contribute constructively to odor and taste with fruity, floral, and fresh fragrances, like 2-butenal, heptanal or octanal, while others are unfavorable expressing a pungent aroma and being toxic at low threshold, like furfural or benzaldehyde[34].

From multivariate analysis, combined drinks fermented with the mixes were addressed by a typical signature made primarily by of 2-heptenal,(Z), hexanal, and octanal. These VOCs are desirable in a beverage, because can confer nice aromatic features. For example, 2-heptenal (Z) is found in bitter cocktails obtained from

fermentation of plant-based material and its odor is described as oily fatty green dairy milky creamy[36]. Octanal has been associated to plant-based fermented drinks and has a still partially green odor, but with clear nuances of citrus[37]; although it is found in orange peel and mandarin[37,38]. While 2-heptenal (Z) is also recognized as an anti-inflammatory and anti-oxidant compound[39,40]. Octanal has properties of antimicrobial activity directed to fungi as those deputed to food spoilage, such as *Pennicillium digitatum* damaging its spore membranes through lipid peroxidation[40], also has antioxidant capacity as radical scavenging activity[37]. Our data evidenced a negative correlation of 2-heptenal (Z) with all bacterial features and in detail significantly with ethanol and acetate production. A similar trend was seen in hemp seed drinks fermented by probiotics[17].

4.2.4. Ketones. Ketones production is a result of bacterial fermentation and lipid oxidation[33]. Some of them are desirable, such as 2-butanone-3-hydroxy, which gives the products a butter aroma with creamy undertones, and 2-nonanone, that have been described to confer several positive sensory/aroma attributes[41]. 2-nonanone has been found in plants, *e.g.* cinnamon, cloves and coconut, in which it showed insecticidal activity in addition to contributing to the flavor[42]. Combined drinks were characterized by a high concentration of 2-pentanone, a flavor volatile compound mainly derived from the enzymatic oxidation of fatty acid and associated with the pungent aroma[43]. In the blends, SR75 had a different profile than SR25 and/or SR50. The former had high concentrations of 7-pentadecanone and 11-dodecen-2-one, while the two latter were plenty of 3-nonanone,2-methyl. The higher concentration of 3,5-dimethyl-2-furyl methyl ketone was associated in particular with soy. In fact, drinks with a higher percentage of soy had a higher concentration of this compound while it decreased by mixing the drinks. This compound although is permitted as a food additive by the main regulatory national and international agencies is a disputed putative toxic substance in higher concentration, as many other furans[44]. Our results showed also that the blends were characterized by a high concentration of 2-butanone,3-hydroxy. This compound is essential in fermented food and confers a pleasant yogurt smell and a butter flavor. Natural sources of this VOC are fruits, vegetables and flours, but it can be biosynthesized in some fermented products. For example, in yogurt and cheese, lactic acid bacteria generate it from lactose and citrate[45]. In fact, our results showed the higher concentration of acetoin in fermented drinks rather than NF.

4.2.5. Organic Acids. Organic acids are mainly produced during fermentation and contribute largely to the definition of the sensorial and nutritional characteristics of the final product. However, not all organic acids bring positive aromatic notes. For example, medium-chain acids, such as hexanoic or octanoic acid, confer a negative rancid taste[34,18] while butanoic acid is a metabolite of controversial sensory properties. In fact, it has been described important to attribute the sour note, like for example in yogurt[46]. In other foods, however, like cider or beer, it is considered a negative compound, responsible for the rancid and cheesy aroma[47].

Our results showed a clear distinction between organic acids which characterized the fermented drinks and those which characterized the NF. In fact, speciation and abundances were larger and higher in fermented drinks. From the multivariate analysis a cluster mainly grouped the samples related to the single fermented drinks, whose signature was made mainly by 5-aminovaleric acid, hypothetically produced through bacterial catabolism of lysine[48].

Our results showed that hexanoic acid, which possess unpleasant flavoring traits (rancid-like)[49,34], decreased in blends rather than in single drinks. Propanoic and lactic acids are flavoring compounds but are also involved in the quality and safety of fermented food due to their antimicrobial activity[17,46]. In fact, lactic acid lowers the pH value creating an unfavorable environment for the development of some pathogens and deteriorating microorganisms[51]. Acetic acid also shows antimicrobial activity against bacilli and antifungal activity[52,53].

Our data evidenced a positive correlation of dihydroxymaleic acid with acidification while a negative correlation was showed with bacterial growth. Presumably, the acidification caused by this acid impeded the growth of lactic acid bacteria and bifidobacteria. On the contrary, heptanoic and nonanoic acid showed a positive correlation both with acidification and with growth and this meant that the acidification caused by these two acids not impeded the bacterial growth.

5. Conclusions

In this work we have demonstrated that to obtain a proper plant-base drink fermented by probiotics the combination of different feedstocks could produce a more effective product, by the mean of; i) faster acidification and buffering capability over time of fermentation; ii) more abundant bacterial growth; iii) abundance of health-related compounds, e.g. butanoic acid; iv) speciation of desirable flavoring compounds, e.g. 2-butanone-3-hydroxy; v) reduction of toxic compounds, e.g. 2-acetyl-3,5-dimethylfuran; vi) reduction of unfavorable stinky compounds, e.g. hexanoic acid.

The plant-based drink market is growing and differentiating rapidly, and new products are necessary, harboring ever healthier and more eco-friendly features. A natural occurring strategy to generate these products could be blending the matrices and fermenting with probiotic strains, which could result in higher functional value, thanks to the production of bioactive compounds, such as SCFA. This work showed that combined drinks better supported the growth of probiotic strains, showing higher bacterial loads than single drinks. Moreover, the blends had a higher abundance of desirable compounds, such as 2-butanone, 3-hydroxy, and a lower amount of undesirable compounds, such as hexanoic acid, which possess unpleasant flavoring traits (rancid-like). This work provides

a useful basis for the formulation of vegetal drinks produced by mixing different plant matrices, exploiting the fermentation process by probiotic strains for the production of bioactive compounds.

Author Contributions

Conceptualization: A.G., L.N.; methodology: A.G., L.N.; software: A.G., F.C., and L.N.; validation: A.G., L.N.; investigation: A.G., F.C., and L.N.; resources: A.G.; data curation: A.G., F.C., and L.N.; writing—original draft preparation: F.C. and L.N.; writing—review and editing: A.G., F.C., and L.N.; supervision: A.G. and L.N.; funding acquisition: A.G.

Conflicts of interest

The authors declare no conflict of interest.

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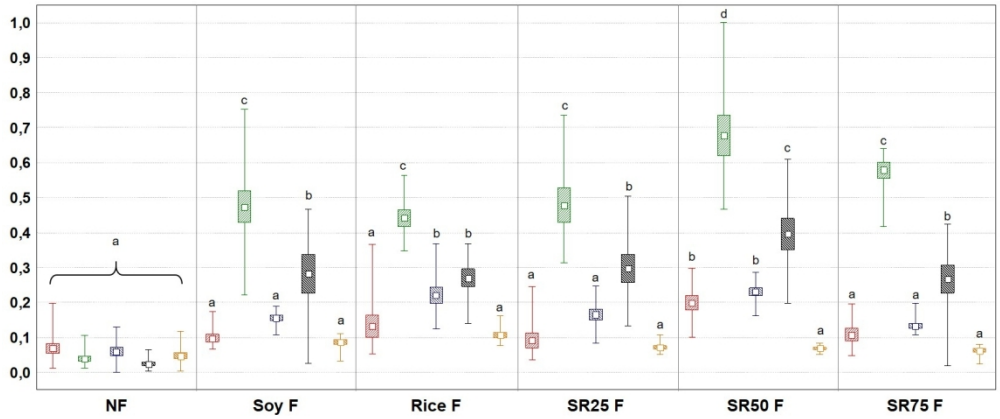

Figure 1. Relative quantification of total volatile organic compounds (VOCs) divided by chemical classes prior and after fermentation. Different letters indicate different significance values by Tukey's HSD (honestly significant difference) test ($P < 0.05$). Sample abbreviations: NF = not fermented samples; F = fermented samples. SR25 = Blend with 25% (v/v) soy drink and 75% (v/v) rice drink; SR50 = Blend with 50% (v/v) soy drink and 50% (v/v) rice drink; SR75 = Blend with 75% (v/v) soy drink and 25% (v/v) rice drink. Square = mean; box = mean ± Standard Deviation (SD); whiskers = max & min. Red plots = aldehydes; green plots = alcohols; blue plots = ketones; black plots = organic acids; yellow plots = others (alkanes, amines, alkenes).

284x123mm (150 x 150 DPI)

Table 1. Means values in mg/Kg of main metabolites from bacterial fermentation of drinks.

Sample	ethyl alcohol	2-butanone,3-hydroxy	acetic acid	lactic acid
S	n.d.	n.d.	n.d.	n.d.
R	n.d.	n.d.	n.d.	n.d.
SR25	n.d.	n.d.	n.d.	n.d.
SR50	n.d.	n.d.	n.d.	n.d.
SR75	n.d.	n.d.	n.d.	n.d.
Slh	12.16 ± 0.02^{c}	tr.	tr.	0.24 ± 0.23^{a}
Slr	25.20 ± 0.02^{d}	0.45 ± 0.04^{b}	1.55 ± 0.04^{b}	0.32 ± 0.04^{a}
Sbb	19.46 ± 0.04^{d}	0.48 ± 0.03^{b}	1.65 ± 0.06^{b}	0.14 ± 0.03^{a}
Sm1	19.46 ± 0.04^{d}	0.48 ± 0.03^{b}	1.65 ± 0.13^{b}	0.14 ± 0.04^{a}
Sbl	13.17 ± 0.09^{c}	0.40 ± 0.04^{b}	0.50 ± 0.08^{a}	0.15 ± 0.03^{a}
Sm2	13.28 ± 0.02^{c}	0.27 ± 0.04^{a}	1.53 ± 0.23^{b}	0.12 ± 0.06^{a}
Rlh	5.63 ± 0.05^{b}	0.52 ± 0.04^{b}	1.45 ± 0.13^{b}	0.17 ± 0.08^{a}
Rlr	12.96 ± 0.45^{c}	0.41 ± 0.01^{b}	1.23 ± 0.12^{b}	0.43 ± 0.08^{b}
Rbb	1.45 ± 0.89^{a}	0.39 ± 0.08^{b}	0.44 ± 0.07^{a}	0.13 ± 0.07^{a}
Rm1	15.70 ± 0.37^{c}	0.31 ± 0.01^{a}	1.56 ± 0.25^{b}	0.018 ± 0.06^{a}
Rm1	15.70 ± 0.37^{c}	0.31 ± 0.01^{a}	1.56 ± 0.25^{b}	tr.
Rbl	1.95 ± 0.18^{a}	0.44 ± 0.04^{b}	0.37 ± 0.15^{a}	tr.
Rm2	6.80 ± 0.40^{b}	0.40 ± 0.03^{b}	1.53 ± 0.26^{b}	tr.
SR25lh	5.63 ± 0.05^{b}	0.52 ± 0.04^{b}	7.23 ± 0.15^{c}	0.87 ± 0.09^{c}
SR25lr	12.96 ± 0.45^{c}	0.41 ± 0.01^{b}	6.15 ± 0.11^{c}	1.67 ± 0.44^{c}
SR25bb	0.45 ± 0.52^{a}	0.39 ± 0.07^{b}	2.22 ± 0.08^{b}	0.56 ± 0.04^{b}
SR25m1	15.70 ± 0.37^{c}	0.31 ± 0.03^{a}	7.78 ± 0.53^{c}	0.91 ± 0.23^{c}
SR25bl	0.57 ± 0.34^{a}	0.44 ± 0.18^{b}	1.85 ± 0.06^{b}	0.44 ± 0.06^{b}
SR25m2	6.80 ± 0.40^{b}	0.40 ± 0.01^{b}	7.64 ± 0.84^{c}	0.41 ± 0.03^{b}
SR50lh	11.67 ± 0.45^{c}	0.51 ± 0.03^{b}	7.84 ± 0.25^{c}	0.96 ± 0.38^{c}
SR50lr	20.25 ± 0.47^{d}	0.59 ± 0.03^{c}	7.14 ± 0.48^{c}	1.24 ± 0.32^{c}
SR50bb	0.52 ± 0.05^{a}	0.90 ± 0.02^{c}	1.87 ± 0.07^{b}	0.54 ± 0.02^{b}
SR50m1	4.94 ± 0.46^{b}	0.75 ± 0.03^{c}	7.65 ± 0.44^{c}	0.70 ± 0.22^{b}
SR50bl	0.22 ± 0.29^{a}	0.71 ± 0.03^{c}	2.55 ± 0.11^{b}	0.56 ± 0.17^{b}
SR50m2	12.03 ± 0.13^{c}	0.48 ± 0.09^{b}	8.06 ± 0.61^{c}	0.80 ± 0.15^{c}
SR75lh	12.16 ± 0.02^{c}	0.36 ± 0.21^{b}	0.22 ± 0.06^{a}	0.66 ± 0.19^{b}
SR75lr	25.20 ± 0.02^{d}	0.45 ± 0.04^{b}	7.77 ± 0.02^{c}	1.08 ± 0.20^{c}
SR75bb	9.46 ± 0.03^{b}	0.48 ± 0.03^{b}	8.24 ± 0.65^{c}	0.71 ± 0.17^{b}
SR75m1	19.46 ± 0.04^{d}	0.48 ± 0.04^{b}	8.24 ± 0.22^{c}	0.72 ± 0.17^{b}

SR75bl	13.17 ± 0.09[c]	0.40 ± 0.06[b]	2.50 ± 0.01[b]	0.70 ± 0.17[b]
SR75m2	13.28 ± 0.02[c]	0.27 ± 0.03[a]	7.64 ± 0.28[c]	0.45 ± 0.03[b]

[a,b,c]Different letters indicate significant difference within a column by Tukey's HSD test ($P < 0.05$).

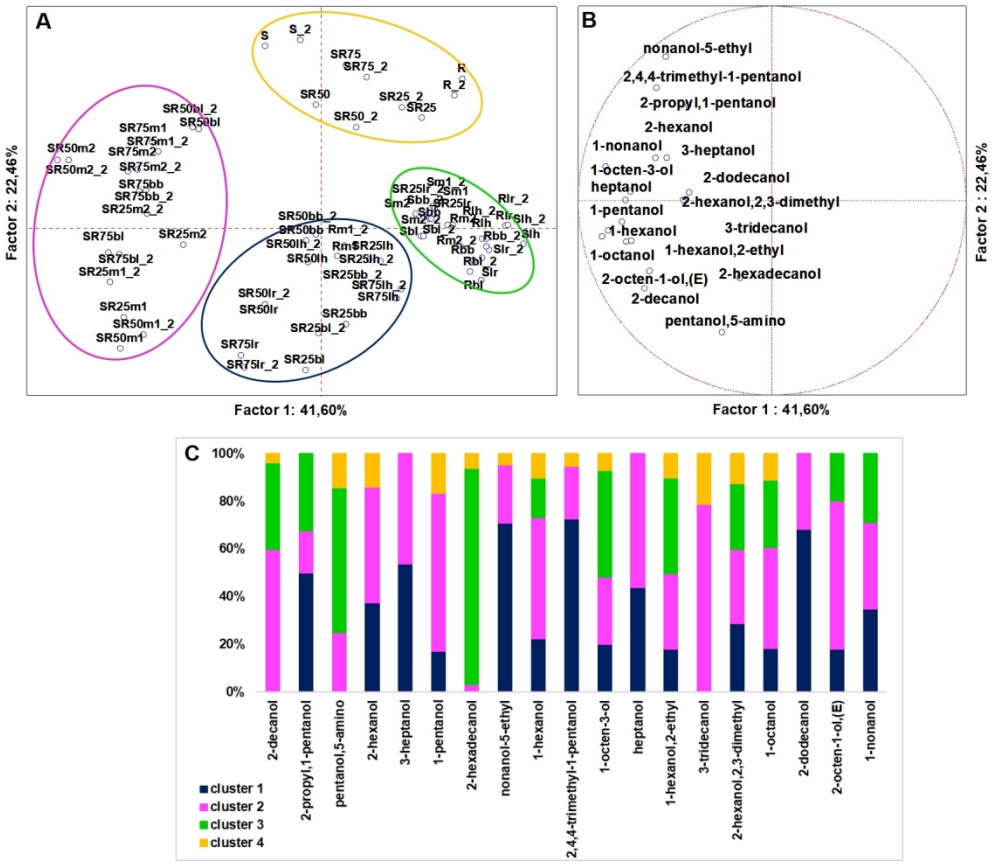

Figure 2. (A) Principal component analysis (PCA) of cases and variables on alcohols ($P < 0.05$); (B) K-means clustering analysis (at least $P < 0.05$).

291x251mm (150 x 150 DPI)

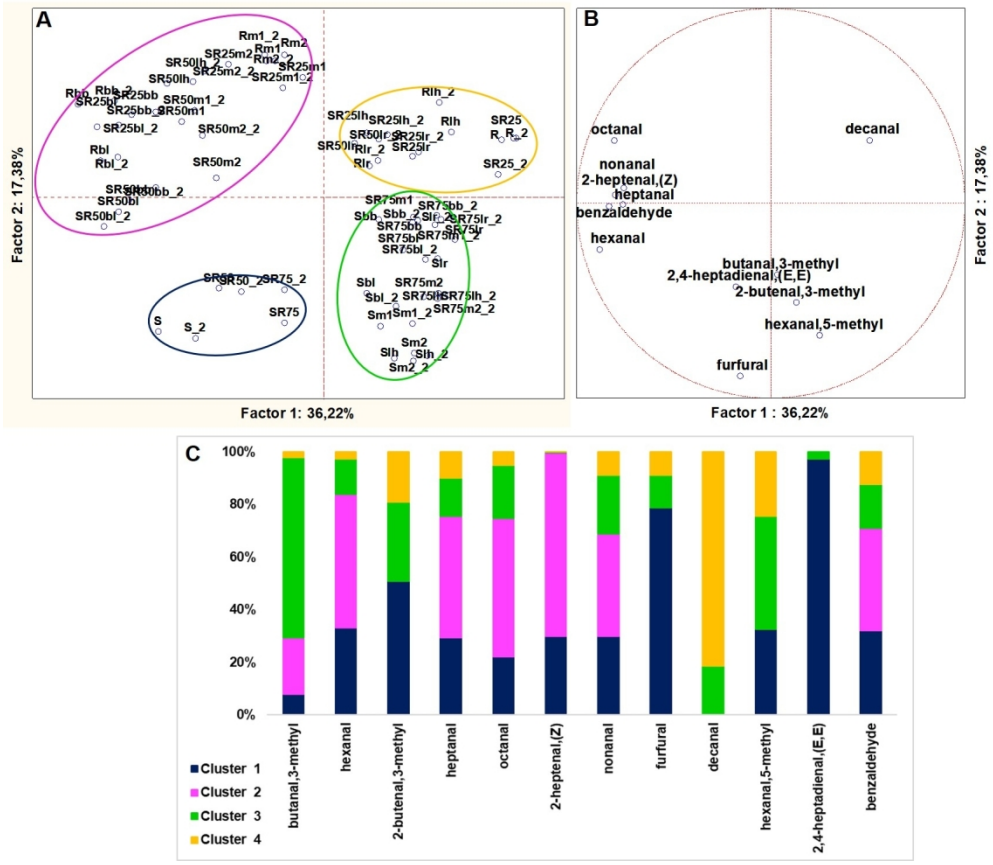

Figure 3. (A) PCA of cases and variables on aldehydes (P < 0.05); (B) K-means clustering analysis (at least P < 0.05).

290x251mm (150 x 150 DPI)

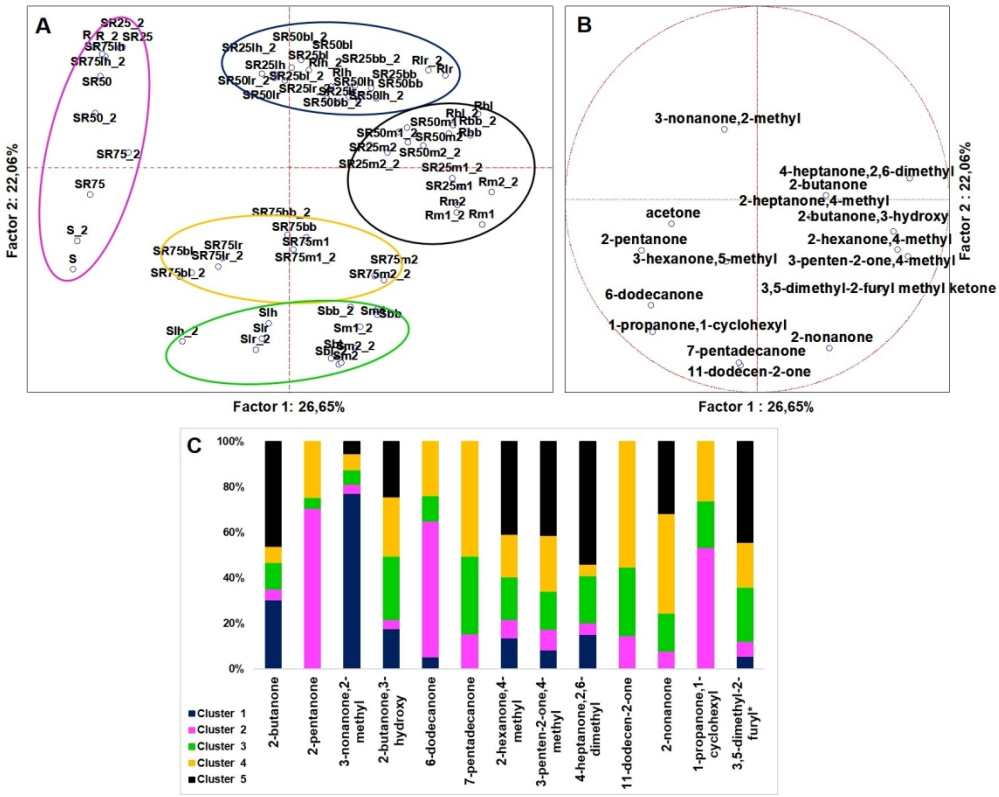

Figure 4. (A) PCA of cases and variables on ketones (P < 0.05); (B) K-means clustering analysis (at least P < 0.05).

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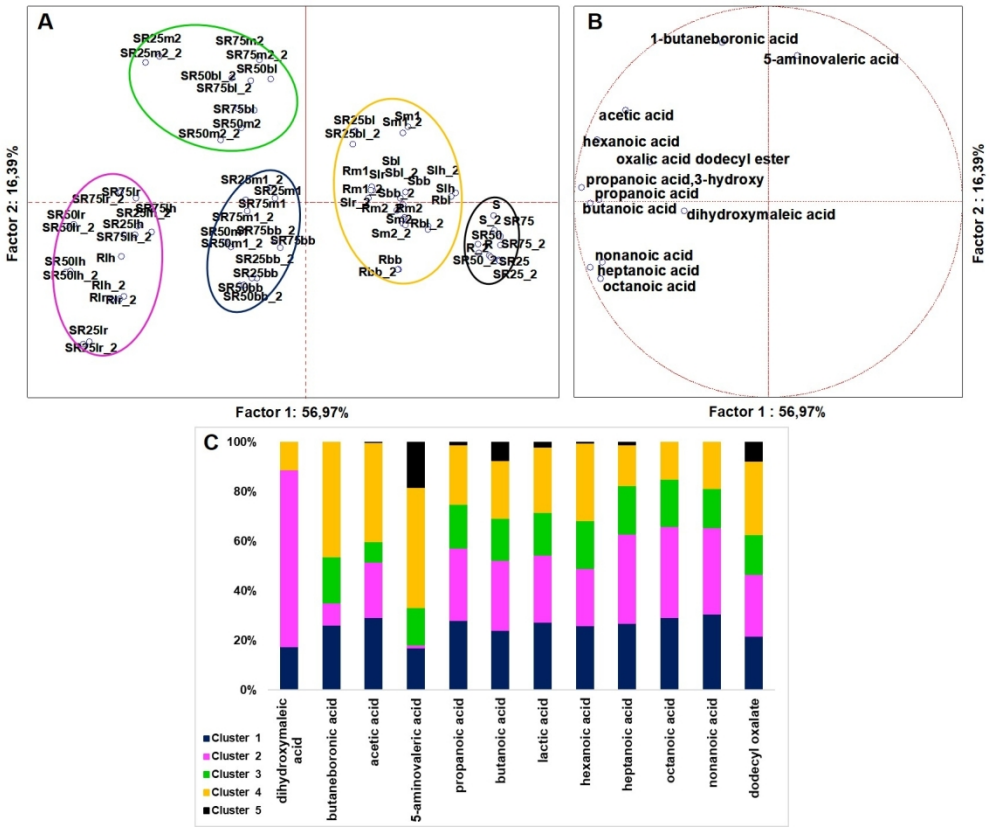

Figure 5. (A) PCA of cases and variables on organic acids (P < 0.05); (B) K-means clustering analysis (P < 0.05). For samples' abbreviations see Table S1 or paragraphs.

290x239mm (150 x 150 DPI)

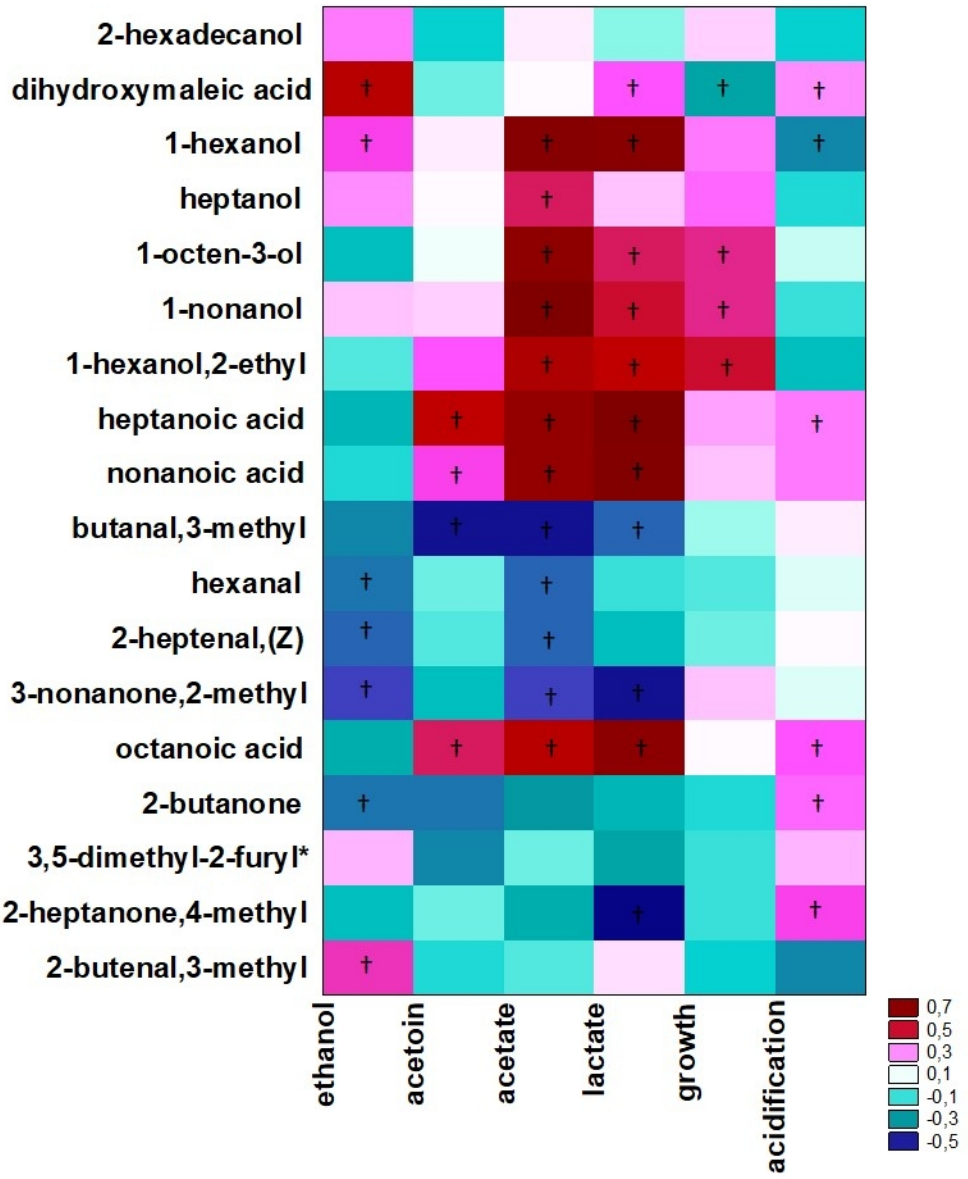

Figure 6. Spearman Rank Correlations Analysis between generation of VOCs and fermentation parameters at the end point of experiments. * 3,5-dimethyl-2-furyl methyl ketone; †Significant correlations ($P < 0.05$).

131x158mm (150 x 150 DPI)

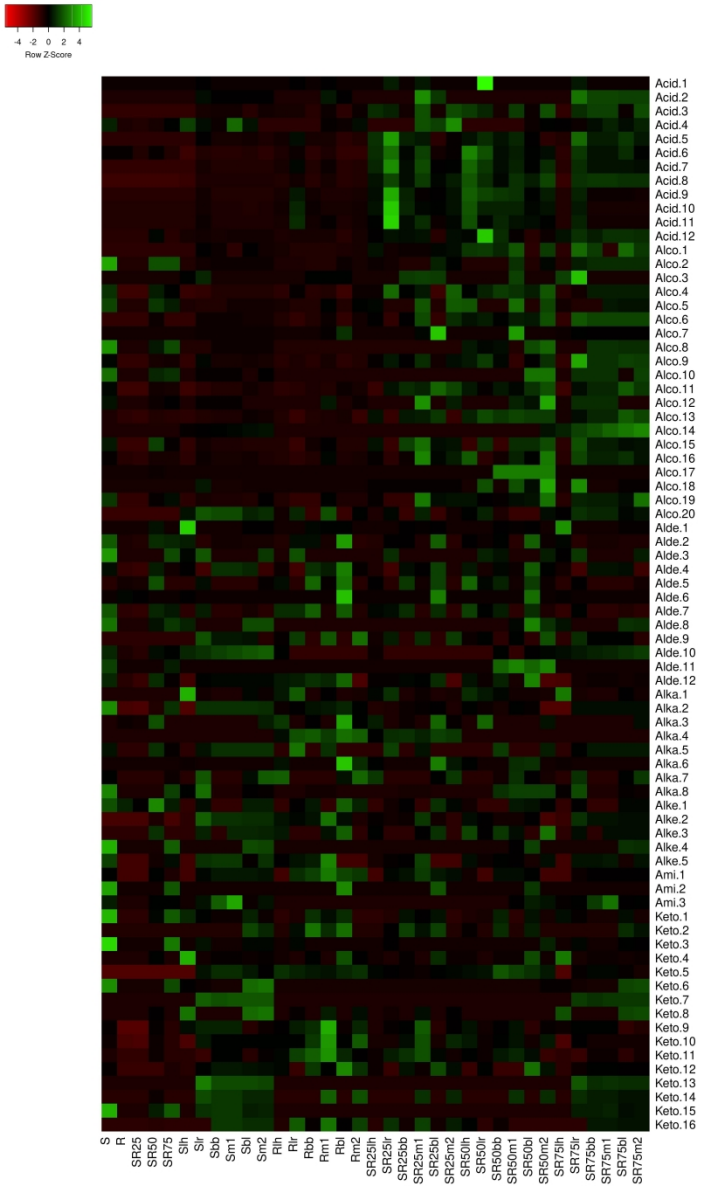

Figure S1. Heatmap of relative quantification of means of VOCs of samples. Acid 1 = dihydroxymaleic acid; Acid 2 = 1-butaneboronic acid; Acid 3 = acetic acid; Acid 4 = 5-aminovaleric acid; Acid 5 = propanoic acid; Acid 6 = butanoic acid; Acid 7 = propanoic acid,3-hydroxy; Acid 8 = hexanoic acid; Acid 9 = heptanoic acid; Acid 10 = octanoic acid; Acid 11 = nonanoic acid; Acid 12 = oxalic acid dodecyl ester; Alcohol 1 = 2-decanol; Alcohol 2 = 2-propyl,1-pentanol; Alcohol 3 = pentanol,5-amino; Alcohol 4 = 2-hexanol; Alcohol 5 = 3-heptanol; Alcohol 6 = 1-pentanol; Alcohol 7 = 2-hexadecanol; Alcohol 8 = nonanol-5-ethyl; Alcohol 9 = 1-hexanol; Alcohol 10 = 2,4,4-trimethyl-1-pentanol; Alcohol 11 = 1-octen-3-ol; Alcohol 12 = heptanol; Alcohol 13 = 1-hexanol,2-ethyl; Alcohol 14 = 3-tridecanol; Alcohol 15 = 2-hexanol,2,3-dimethyl; Alcohol 16 = 1-octanol; Alcohol 17 = 2-dodecanol; Alcohol 18 = 2-octen-1-ol,(E); Alcohol 19 = 1-nonanol; Alcohol 20 = ethyl alcohol; Aldehyde 1 = butanal,3-methyl; Aldehyde 2 = hexanal; Aldehyde 3 = 2-butenal,3-methyl; Aldehyde 4 = heptanal; Aldehyde 5 = octanal; Aldehyde 6 = 2-heptenal,(Z); Aldehyde 7 = nonanal; Aldehyde 8 = furfural; Aldehyde 9 = decanal; Aldehyde 10 = hexanal,5-methyl; Aldehyde 11 = 2,4-heptadienal,(E,E); Aldehyde 12 = benzaldehyde; Alkane 1 = furan,2-methyl; Alkane 2 = furan,2-pentyl; Alkane 3 = decane; Alkane 4 = heptane,2,4-dimethyl; Alkane 5 = dodecane,1-fluoro; Alkane 6 = decane-

3,6-dimethyl; Alkane 7 = nonane; Alkane 8 = octane,2,6-dimethyl; Alkene 1 = 2-octene,(Z); Alkene 2 = 2-pentene,1-butoxy; Alkene 3 = 1-pentene,2,4,4-trimethyl; Alkene 4 = 1 -pentadecene; Alkene 5 = 2-pentene,3-ethyl-2-methyl; Amine 1 = 6-azathymine; Amine 2 = 2-formylhistamine; Amine 3 = acetamide,2-(2,-hydroxyethoxy); Ketone 1 = acetone; Ketone 2 = 2-butanone; Ketone 3 = 2-pentanone; Ketone 4 = 3-nonanone,2-methyl; Ketone 5 = 2-butanone,3-hydroxy; Ketone 6 = 6-dodecanone; Ketone 7 = 7-pentadecanone; Ketone 8 = 3-hexanone,5-methyl; Ketone 9 = 2-hexanone,4-methyl; Ketone 10 = 3-penten-2-one,4-methyl; Ketone 11 = 4-heptanone,2,6-dimethyl; Ketone 12 = 2-heptanone,4-methyl; Ketone 13 = 11-dodecen-2-one; Ketone 14 = 2-nonanone; Ketone 15 = 1-propanone,1-cyclohexyl; Ketone 16 = 3,5-dimethyl-2-furyl methyl ketone.

285x464mm (300 x 300 DPI)

Supplementary Table 1

Sample	Description
Slh	Soy drink* fermented with 5 Log_{10} cells/ml load of *Lactobacillus helveticus* CBNL (lh)
Slr	Soy drink fermented with 5 Log_{10} cells/ml load of *L. rhamnosus* C243 (lr)
Sbb	Soy drink fermented with 5 Log_{10} cells/ml load of *Bifidobacterium. bifidum* B700795 (bb)
Sm1	Soy drink fermented with 5 Log_{10} cells/ml load of a mix equally containing lh, lr, and bb (m1)
Sbl	Soy drink fermented with 5 Log_{10} cells/ml of *Bifidobacterium. longum* Bl12 (bl)
Sm2	Soy drink fermented with 5 Log_{10} cells/ml of a mix equally containing lh, lr, bb, and bl (m2)
Rlh	Rice drink** fermented with 5 Log_{10} cells/ml load of lh
Rlr	Rice drink fermented with 5 Log_{10} cells/ml load of lr
Rbb	Rice drink fermented with 5 Log_{10} cells/ml load of bb
Rm1	Rice drink fermented with 5 Log_{10} cells/ml load of m1
Rbl	Rice drink fermented with 5 Log_{10} cells/ml load of bl
Rm2	Rice drink fermented with 5 Log_{10} cells/ml load of m2
SR25lh	Soy/Rice (25/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of lh
SR25lr	Soy/Rice (25/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of lr
SR25bb	Soy/Rice (25/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of bb
SR25m1	Soy/Rice (25/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of m1
SR25bl	Soy/Rice (25/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of bl
SR25m2	Soy/Rice (25/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of m2
SR50lh	Soy/Rice (50/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of lh
SR50lr	Soy/Rice (50/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of lr
SR50bb	Soy/Rice (50/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of bb
SR50m1	Soy/Rice (50/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of m1
SR50bl	Soy/Rice (50/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of bl
SR50m2	Soy/Rice (50/75 % v/v) drink fermented with 5 Log_{10} cells/ml load of m2
SR75lh	Soy/Rice (75/25 % v/v) drink fermented with 5 Log_{10} cells/ml load of lh
SR75lr	Soy/Rice (75/25 % v/v) drink fermented with 5 Log_{10} cells/ml load of lr
SR75bb	Soy/Rice (75/25 % v/v) drink fermented with 5 Log_{10} cells/ml load of bb
SR75m1	Soy/Rice (75/25 % v/v) drink fermented with 5 Log_{10} cells/ml load of m1
SR75bl	Soy/Rice (75/25 % v/v) drink fermented with 5 Log_{10} cells/ml load of bl
SR75m2	Soy/Rice 75/25 % v/v) drink fermented with 5 Log_{10} cells/ml load of m2

Supplementary Table 2. Quantification means of bacterial cells during fermentation of drinks.

Sample	Log_{10} cells/ml			Delta Log_{10} cells/ml	
	0 h	*6 h*	*24 h*	*6 h*	*24 h*
Slh	7.12 ± 0.14[a]	8.45 ± 0.29[b]	9.87 ± 0.23[d]	1.33 ± 0.13[†]	2.75 ± 0.36[†]
Slr	7.14 ± 0.18[a]	8.71 ± 0.32[b]	9.33 ± 0.31[c]	1.57 ± 0.11[†]	2.19 ± 0.19[††]
Sbb	7.04 ± 0.11[a]	8.61 ± 0.24[b]	9.63 ± 0.36[c]	1.57 ± 0.13[**]	2.59 ± 0.12[†]
Sm1	7.10 ± 0.27[a]	8.89 ± 0.21[b]	10.20 ± 0.32[d]	1.79 ± 0.11[**]	3.10 ± 0.13[††]
Sbl	7.09 ± 0.09[a]	8.30 ± 0.25[b]	9.19 ± 0.21[c]	1.21 ± 0.20[**]	2.10 ± 0.26[†]
Sm2	7.14 ± 0.22[a]	8.51 ± 0.18[b]	10.11 ± 0.24[d]	1.37 ± 0.15[**]	2.97 ± 0.38[††]
Rlh	7.17 ± 0.14[a]	7.98 ± 0.19[a]	9.16 ± 0.26[c]	0.81 ± 0.09[*]	1.99 ± 0.24[†]
Rlr	7.30 ± 0.25[a]	8.20 ± 0.23[b]	9.11 ± 0.20[c]	0.90 ± 0.06[**]	1.81 ± 0.14[**]
Rbb	7.28 ± 0.07[a]	7.82 ± 0.19[a]	9.83 ± 0.22[d]	0.54 ± 0.12[*]	2.55 ± 0.10[†]
Rm1	7.32 ± 0.17[a]	7.97 ± 0.15[a]	9.77 ± 0.18[c]	0.65 ± 0.18[*]	2.45 ± 0.12[†]
Rbl	7.25 ± 0.21[a]	8.54 ± 0.22[b]	10.05 ± 0.28[d]	1.29 ± 0.21[**]	2.80 ± 0.16[†]
Rm2	7.13 ± 0.32[a]	8.14 ± 0.30[b]	9.97 ± 0.22[d]	1.01 ± 0.22[**]	2.84 ± 0.23[†]
SR25lh	7.03 ± 0.14[a]	9.15 ± 0.22[c]	9.62 ± 0.19[c]	2.12 ± 0.11[†]	2.59 ± 0.21[†]
SR25lr	7.05 ± 0.23[a]	9.88 ± 0.15[d]	9.68 ± 0.21[c]	2.83 ± 0.12[†]	2.63 ± 0.34[†]
SR25bb	6.98 ± 0.13[a]	8.48 ± 0.26[b]	10.17 ± 0.24[d]	1.50 ± 0.12[**]	3.19 ± 0.23[††]
SR25m1	6.97 ± 0.20[a]	9.04 ± 0.15[b]	9.86 ± 0.14[d]	2.07 ± 0.18[†]	2.89 ± 0.28[†]
SR25bl	7.02 ± 0.12[a]	9.28 ± 0.17[c]	9.45 ± 0.15[c]	2.26 ± 0.15[†]	2.43 ± 0.25[†]
SR25m2	7.08 ± 0.25[a]	9.45 ± 0.18[c]	9.88 ± 0.16[d]	2.37 ± 0.08[†]	2.82 ± 0.31[†]
SR50lh	7.01 ± 0.12[a]	9.65 ± 0.14[c]	10.09 ± 0.21[d]	2.64 ± 0.08[†]	3.08 ± 0.32[††]
SR50lr	6.95 ± 0.16[a]	9.34 ± 0.07[c]	9.71 ± 0.22[c]	2.39 ± 0.15[**]	2.76 ± 0.13[†]
SR50bb	7.13 ± 0.19[a]	8.30 ± 0.22[b]	10.18 ± 0.29[d]	1.17 ± 0.09[**]	3.05 ± 0.08[††]
SR50m1	7.07 ± 0.11[a]	9.36 ± 0.31[c]	10.15 ± 0.14[d]	2.29 ± 0.13[**]	3.08 ± 0.19[††]
SR50bl	6.97 ± 0.13[a]	9.83 ± 0.30[d]	10.03 ± 0.13[d]	2.86 ± 0.19[†]	3.06 ± 0 20[††]
SR50m2	7.17 ± 0.17[a]	9.87 ± 0.12[d]	9.98 ± 0.31[d]	2.70 ± 0.11[**]	2.81 ± 0.19[†]
SR75lh	7.09 ± 0.12[a]	9.84 ± 0.22[d]	10.03 ± 0.18[d]	2.75 ± 0.27[†]	2.94 ± 0.16[†]
SR75lr	6.98 ± 0.19[a]	9.43 ± 0.32[c]	9.97 ± 0.09[d]	2.45 ± 0.22[**]	2.99 ± 0.28[††]
SR75bb	6.96 ± 0.18[a]	8.30 ± 0.10[b]	10.33 ± 0.23[d]	1.34 ± 0.07[*]	3.37 ± 0.23[††]
SR75m1	7.13 ± 0.28[a]	9.36 ± 0.22[c]	10.37 ± 0.35[d]	2.23 ± 0.10[**]	3.24 ± 0.32[††]
SR75bl	7.04 ± 0.17[a]	9.85 ± 0.11[d]	9.90 ± 0.17[d]	2.81 ± 0.22[†]	2.86 ± 0.27[†]
SR75m2	7.11 ± 0.12[a]	9.11 ± 0.12[c]	10.05 ± 0.27[d]	2.03 ± 0.18[**]	2.94 ± 0.16[†]

Supplementary Table 3. pH mean value of drinks during fermentation

Sample	p H			Delta pH	
	0 h	*6 h*	*24 h*	*6 h*	*24 h*
Slh	7.14 ± 0.04[a]	5.15 ± 0.19[b]	4.27 ± 0.09[b]	1.99 ± 0.11[†]	2.87 ± 0.06[†]
Slr	7.10 ± 0.07[a]	5.30 ± 0.21[b]	4.13 ± 0.11[b]	1.80 ± 0.14[†]	2.97 ± 0.09[††]

Sbb	7.04 ± 0.11[a]	6.01 ± 0.14[a]	4.21 ± 0.16[b]	1.03 ± 0.13[**]	2.83 ± 0.14[†]
Sm1	7.10 ± 0.19[a]	5.49 ± 0.18[a]	4.20 ± 0.12[b]	1.61 ± 0.18[**]	2.90 ± 0.15[††]
Sbl	7.09 ± 0.31[a]	5.92 ± 0.18[a]	3.99 ± 0.22[c]	1.17 ± 0.24[**]	3.10 ± 0.26[††]
Sm2	7.14 ± 0.52[a]	5.60 ± 0.18[a]	4.15 ± 0.14[b]	1.54 ± 0.35[**]	2.99 ± 0.33[††]
Rlh	7.27 ± 0.03[a]	4.88 ± 0.16[b]	3.16 ± 0.06[d]	2.39 ± 0.09[†]	4.10 ± 0.04[††]
Rlr	7.30 ± 0.05[a]	5.10 ± 0.27[b]	3.01 ± 0.23[d]	2.20 ± 0.16	4.29 ± 0.14[††]
Rbb	7.28 ± 0.08[a]	5.52 ± 0.16[a]	3.63 ± 0.12[c]	1.76 ± 0.12[**]	3.65 ± 0.10[††]
Rm1	7.32 ± 0.14[a]	5.17 ± 0.20[b]	3.27 ± 0.14[c]	2.15 ± 0.17[†]	4.05 ± 0.14[††]
Rbl	7.25 ± 0.24[a]	5.44 ± 0.19[b]	3.69 ± 0.08[c]	1.81 ± 0.21[†]	3.56 ± 0.16[††]
Rm2	7.32 ± 0.39[a]	5.24 ± 0.19[b]	3.37 ± 0.12[c]	2.09 ± 0.29[†]	3.95 ± 0.26[††]
SR25lh	6.93 ± 0.21[a]	4.28 ± 0.02[b]	3.71 ± 0.11[c]	2.65 ± 0.11[†]	3.22 ± 0.11[††]
SR25lr	6.95 ± 0.31[a]	5.45 ± 0.01[b]	4.06 ± 0.01[c]	1.50 ± 0.16[**]	2.89 ± 0.16[†]
SR25bb	6.97 ± 0.02[a]	6.84 ± 0.02[a]	3.87 ± 0.01[c]	0.13 ± 0.02[*]	3.10 ± 0.01[††]
SR25m1	6.95 ± 0.21[a]	5.72 ± 0.05[a]	3.74 ± 0.05[c]	1.23 ± 0.13[**]	3.21 ± 0.11[††]
SR25bl	6.98 ± 0.09[a]	4.73 ± 0.02[b]	3.73 ± 0.01[c]	2.25 ± 0.05[†]	3.25 ± 0.05[††]
SR25m2	6.98 ± 0.02[a]	5.03 ± 0.03[b]	3.71 ± 0.04[c]	1.95 ± 0.02[†]	3.27 ± 0.01[††]
SR50lh	6.91 ± 0.04[a]	4.51 ± 0.06[b]	3.93 ± 0.11[c]	2.40 ± 0.05[†]	2.98 ± 0.02[††]
SR50lr	6.92 ± 0.06[a]	5.60 ± 0.05[a]	4.15 ± 0.02[b]	1.32 ± 0.05[**]	2.77 ± 0.04[†]
SR50bb	6.93 ± 0.09[a]	6.85 ± 0.02[a]	4.15 ± 0.09[b]	0.09 ± 0.05[*]	2.79 ± 0.06[†]
SR50m1	6.97 ± 0.14[a]	5.95 ± 0.02[a]	3.87 ± 0.13[c]	1.02 ± 0.08[**]	3.10 ± 0.09[††]
SR50bl	6.98 ± 0.23[a]	5.05 ± 0.03[b]	3.67 ± 0.11[c]	1.93 ± 0.13[†]	3.31 ± 0.12[††]
SR50m2	6.97 ± 0.17[a]	5.32 ± 0.02[b]	3.74 ± 0.32[c]	1.65 ± 0.10[**]	3.23 ± 0.09[††]
SR75lh	6.89 ± 0.12[a]	4.64 ± 0.01[b]	3.97 ± 0.08[c]	2.25 ± 0.07[†]	2.92 ± 0.06[†]
SR75lr	6.95 ± 0.13[a]	5.77 ± 0.28[a]	4.39 ± 0.03[b]	1.18 ± 0.21[**]	2.56 ± 0.08[†]
SR75bb	6.93 ± 0.05[a]	6.91 ± 0.07[a]	4.37 ± 0.09[b]	0.02 ± 0.06[*]	2.56 ± 0.03[†]
SR75m1	6.93 ± 0.09[a]	6.11 ± 0.16[a]	3.90 ± 0.11[c]	0.82 ± 0.12[**]	3.03 ± 0.05[††]
SR75bl	6.94 ± 0.14[a]	5.32 ± 0.11[b]	3.84 ± 0.08[c]	1.62 ± 0.12[**]	3.10 ± 0.07[††]
SR75m2	6.99 ± 0.11[a]	5.68 ± 0.09[a]	3.82 ± 0.02[c]	1.31 ± 0.10[**]	3.16 ± 0.06[††]