Use of Reconstituted Kefir Consortia to Determine the Impact of Microbial Composition on Kefir Metabolite Profiles

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Highlights

- Traditional kefir has more yeast associated metabolites than a reconstituted kefir
- Altering microbial composition of the kefir consortium changes metabolite profile
- Lactobacilli drive organic acid production in kefir consortium fermentations

Keywords: Fermentation Dynamics, Synthetic microbial communities, Metabolomics, Fermented Foods, Lactobacillus, Dairy

Abstract

Kefir is fermented traditionally with kefir grains but commercial kefir production often relies on fermentation with planktonic cultures. Kefir has been associated with many health benefits, however, the utilization of kefir grains to facilitate large industrial production of kefir is challenging and makes to difficult to ensure consistent product quality and consistency. Notably, the microbial composition of kefir fermentations has been shown to impact kefir associated health benefits. This study aimed to compare volatile compounds, organic acids, and sugar composition of kefir produced through a traditional grain fermentation and through a reconstituted kefir consortium fermentation. Additionally, the impact of two key microbial communities on metabolite production in kefir was assessed using two modified versions of the consortium, with either yeasts or lactobacilli removed. We hypothesized that the complete kefir consortium would closely resemble traditional kefir, while the consortia without yeasts or lactobacilli would differ significantly from both traditional kefir and the complete consortium fermentation. Kefir fermentations were examined after 12 and 18 h using two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-TOFMS) to identify volatile compounds and high performance liquid chromatography (HPLC) to identify organic acid and sugar composition. The traditional kefir differed significantly from the kefir consortium fermentation with the traditional kefir having 15-20 $\log_2$(fold change) higher levels of esters and the consortium fermented kefir having between 1-3 $\log_2$(fold change) higher organic acids including lactate and acetate. The use of a version of kefir consortium that lacked lactobacilli resulted in between 2 and 20 $\log_2$(fold change) lower levels of organic acids, ethanol, and butanoic acid ethyl ester, while the absence of yeast from the consortium resulted in minimal change. In summary, the kefir consortium fermentation is significantly different from traditional grain fermented kefir with respect to the

profile of metabolites present, and seems to be driven by lactobacilli, as evidenced by the significant decrease in multiple metabolites when the lactobacilli were removed from the fermentation and minimal differences observed upon the removal of yeast.

Abbreviations

GC×GC-TOFMS, two-dimensional gas chromatography-time-of-flight mass spectrometry; HPLC, high performance liquid chromatography; MRS, De Man, Rogosa, and Sharpe; YEGC, yeast extract, glucose, and chloramphenicol; SPME, solid-phase microextraction; DVB/CAR/PDMS, Divinylbenzene/Carboxen/Polydimethylsiloxane; PCA, principal component analysis; ADONIS, Analysis of Variance using Distance Matrices; COX, cyclooxygenase

1. Introduction

The fermented dairy beverage kefir has been consumed for hundreds of years. It originated in the Caucasus region of Europe but gained popularity worldwide. Kefir is relatively unique when compared to other fermented dairy products as it is fermented by a floating biofilm that contains a diverse community of yeasts, fungi, lactic acid bacteria and acetic acid bacteria resulting in a product with a unique and complex profile of volatile metabolites and organic acids (Bourrie et al., 2016; Walsh et al., 2016). While traditional microbial communities in kefir grains and grain-fermented kefir can differ significantly in their microbial composition, the core microbiome and metabolite profile of these fermentations is relatively consistent (Blasche et al., 2021; Cui et al., 2023; Dobson et al., 2011; Marsh et al., 2013; Nejati et al., 2022; Walsh et al., 2016). Traditional kefirs often differ significantly from kefir that is produced commercially in both microbial composition and metabolite profile. Commercial kefir is frequently produced with planktonic cells while traditional kefir fermentation is initiated with a surface associated biofilm, the kefir grain. The microbial diversity of commercial cultures is lower than the diversity of kefir grains, and rarely contains yeast species. In contrast, traditional kefir frequently contains a high abundance of *Lactobacillus kefiranofaciens* or other kefir specific lactobacilli and additionally can contain a wide variety of yeast species, which may lead to higher levels of alcohols and esters when compared to commercial kefir (Cui et al., 2023; Nejati et al., 2022; Walsh et al., 2016). Indeed, the addition of kefir specific lactobacilli such as *Lactobacillus kefiranofaciens* can significantly alter the metabolite profile when supplemented into traditional fermentations, resulting in increased levels of esters (Walsh et al., 2016). While there have been previous studies examining the metabolite profile of kefir, these studies have focused solely on grain fermented kefir, pitched

culture kefir, or kefir produced using different milk products or fermentation substrates (Abadl et al., 2023; Cui et al., 2023; Guangsen et al., 2021; Oliveira Filho et al., 2023).

An improved understanding of the kefir fermentation and the development of starter cultures for commercial kefir production is dependent on the successful reconstruction of the communities in traditional kefir grains to analyse fermentations with a controlled community of microbes (Blasche et al., 2021). We previously developed a reconstituted kefir consortium which contains 5 bacterial and 4 yeast species isolated from a single kefir grain (Bourrie et al., 2021). Together, these 9 species represent the core microbes present in kefir produced by this grain, totalling greater than 90% of the microbial population observed in this kefir using shotgun metagenomic sequencing (Walsh et al., 2016). This kefir consortium has comparable cholesterol lowering effects as the traditional kefir from which the organisms were isolated and some of these benefits are maintained in a filter sterilized cell free version of the product (Bourrie et al., 2021, 2022). We have also observed that exclusion of either the lactobacilli or the yeast populations from the fermentation these benefits are completely lost (Bourrie et al., 2021). Together these findings indicate that at least some of the beneficial effects of both traditional kefir and the kefir consortium are likely due to metabolites present in the product and that microbial composition is a key factor in kefir associated health benefits. These findings are in agreement with past work that has identified multiple health benefits associated with kefir metabolites. These benefits include immunomodulatory effects and improvements to cholesterol and lipid metabolism and have been associated with potentially bioactive components in kefir including lactate, peptides, and the exopolysaccharide kefiran (Bourrie et al., 2020; M. C. Tung et al., 2020; Y.-T. Tung et al., 2017; Vieira et al., 2021).

This study aimed to determine whether a grain fermented traditional kefir and a kefir made using a reconstituted consortium of core kefir organisms isolated from said traditional kefir are similar in metabolic properties using metabolomics. We also examined how the removal of the lactobacilli and yeast populations impacted the metabolite profile of the kefir consortium to identify how these organisms contribute to kefir fermentation and how these contributions may impact the health benefits of kefir. In order to achieve this, we performed fermentations with each type of kefir inoculum utilizing three biological repeats for each and sampled fermentations at 12 and 18 hours post-inoculation. Volatile metabolite profiles were then analyzed with two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-TOFMS), while short chain organic acids and sugars were quantified using high performance liquid chromatography (HPLC). To our knowledge, this is the first study to utilize a reconstituted kefir consortium consisting of organisms representing the core microbes present in a single traditional grain fermented kefir and to compare this consortium to the kefir from which it was isolated providing novel insights into the importance of the kefir grain complex in fermentation dynamics. This is also the first study to examine metabolite profiles in multiple kefir fermentations with demonstrated differences in their health benefits, allowing us to identify potential metabolites of importance to kefir's purported health promoting effects.

2. Materials and Methods

2.1 Kefir Production

Kefir production was performed as previously described (Bourrie et al., 2021). Briefly, kefir consortia were prepared by inoculating freshly opened pasteurized 2% fat milk with *Acetobacter pasteurianus* KC001, *Lactococcus lactis* KC002, *Leuconostoc mesenteroides* KC003, *Lentilactobacillus kefiri* KC004, *Lactobacillus kefiranofaciens* KC005, *Pichia fermentans* KC006,

Saccharomyces cerevisiae KC007, *Kazachstania unispora* KC008, and *Kluyveromyces marxianus* KC009. The kefir consortium without lactobacilli was fermented with the same microorganisms but *Lentilactobacillus kefiri* and *Lactobacillus kefiranofaciens* were omitted. The kefir consortium without yeast was fermented with all bacterial members of the kefir consortium but *Pichia fermentans*, *Saccharomyces cerevisiae*, *Kazachstania unispora*, and *Kluyveromyces marxianus* were omitted. Bacterial isolates were plated on De Man, Rogosa, and Sharpe (MRS) agar, while yeast isolates were plated on yeast extract, glucose, and chloramphenicol (YEGC) agar. A single colony was picked into MRS or YEGC broth as appropriate and overnight cultures were inoculated in milk at a starting concentration of 10^5 colony forming units (CFU)/mL of bacteria and 10^4 CFU/mL of yeast. Traditional kefir grains were used to inoculate pasteurized 2% fat milk at 10% weight/volume. Fermentation was carried out in sterile glass jars at room temperature (22℃) for 18 h. These grains have previously undergone metagenomic sequencing to determine their composition (Walsh et al., 2016). Fermentations were performed in triplicate, and 5-mL samples were taken after 12 and 18 h of fermentation and stored at -20 °C until GC×GC-TOFMS and HPLC analysis.

2.2 GC×GC-TOFMS Analysis

The volatiles of kefir samples were extracted via headspace solid-phase microextraction (SPME) using an automated SPME module (Gerstel, Linthicum, MD) and analyzed by a LECO Pegasus 4D GC×GC-TOFMS (Leco Instruments, St. Joseph, MI). Approximately 0.5 g kefir was placed into a 20-mL headspace vial (Gerstel) and kept on ice until 2 minutes before analysis. Vials were incubated at 75 °C for 5 min, followed by 20 minutes of extraction using a 50/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fibre (SUPELCO, Bellefonte, PA). Samples were desorbed for 3 minutes at 250 °C in splitless mode. The column set

used for the two-dimensional chromatography was a 60 m × 0.25 mm × 0.25 µm Rtx-5Sil MS (Chromatographic Specialties) for the first dimension and a 1.6 m × 0.25 mm × 0.25 µm Rtx-200MS (Chromatographic Specialties) for the second-dimension. The initial oven temperature of 80 °C was held for 3 min, followed by a ramp of 3.5 °C /min to 240 °C for a total run time of 50 min. The secondary oven and the modulator were programmed to have a constant offset of +10 °C and +15 °C, relative to the primary and secondary oven temperature, respectively with a modulation period of 2.5 s. The constant flow of 2 mL/min helium (high purity 5.0, Praxair, CA) was used as a carrier gas for the entire run. Mass spectra were acquired over a range of m/z 40-800 with an acquisition rate of 200 Hz. The ion source temperature was set at 200 °C, while the transfer line temperature was set at 250 °C. The detector voltage was set with an offset of -200 V relative to the tuning potential. The acquired chromatograms were processed using LECO ChromaTOF® software (version 4.71.0.0) with a data processing method to find all peaks with S/N>100. Statistical Compare tools in ChromaTOF® were used to align the peaks from all samples based on retention times and mass spectra.

The peak tables present the values of S/N and Area for each peak. Peak areas were normalized by the weight of the sample. Compounds were tentatively identified following alignment using a similarity threshold of 600 and comparison of retention indices to library values (NIST 17 and Wiley 9th edition).

2.3 HPLC Analysis

Organic acids and sugars were analyzed using an Agilent 1200 series HPLC system equipped with an Aminex HPX-87H Column (300 × 7.8 mm; 9 µm, Bio-rad, USA). Samples were eluted at a flow rate of 0.4 ml/min with 5 mM H_2SO_4 as the mobile phase. Quantification was performed on a refractive index (RI) detector and a UV detector (210 nm) using external standards.

2.4 Microbial Density and pH of Kefir

The pH of kefir samples was measured with a glass electrode pH meter. Microbial density for both bacteria and yeast were determined by surface plating of serial 10-fold dilutions in phosphate buffered saline on MRS or YEGC media respectively followed by plate counting to determine CFU/mL in kefir.

2.5 Data Analysis

Comparisons of fermentation types at 18 h, and comparisons within fermentation at 12 and 18 h were performed using Metaboanalyst. Briefly, normalized data was range scaled, and a t-test was performed. Volcano plots were generated for all compounds with a false discovery rate (FDR)-adjusted p-value < 0.10. Principal component analysis (PCA) was employed as an unsupervised method to visualize the overall metabolite profiles generated in the different kefir preparations. Intensity data was analyzed using Analysis of Variance with Tukey post-hoc for multiple comparisons utilizing the R packages multcompView, ggplot2, plyr, and lmPerm. Effect of fermentation type on metabolite composition was determined using Permutational Multivariate Analysis of Variance using Distance Matrices (ADONIS).

3. Results

3.1 Microbial cell counts and pH in traditional kefir and reconstituted kefir consortia.

The three kefir consortia did not differ from the traditional kefir with respect to total bacterial levels or pH, whileno yeast was detected when the consortium without yeast was used (Table 1).

3.2 Kefir Volatile Metabolites Cluster by Fermentation Time and Microbial Composition

PCA analysis of kefir metabolite profiles showed that there was a significant difference in metabolite profiles of different fermentations after 12h (ADONIS = 0.001, Figure 1). After 18 h

of fermentation, samples from kefir that was inoculated with different cultures clustered separately (ADONIS = 0.003, Figure 1). The volatile metabolite profiles of samples obtained at 12 and 18 h from the same kefir also clustered separately (ADONIS = 0.001, Figure 1). Among the kefirs produced using different kefir consortia, samples fermented with the complete kefir consortium for 18 h clustered more closely than every other group, indicating an apparent lower level of variation between samples. Interestingly, the kefir fermented with the complete kefir consortium was no more similar to grain fermented kefir than the fermentations produced by consortia that lacked yeasts or lactobacilli.

3.3. Changes to kefir microbial composition impact metabolite profiles.

A total of 299 compounds were detected by GC×GC-TOFMS, of which 64% (191 compounds) were tentatively identifiable by name based on mass and retention index. Of these, 273 compounds were detected after 18 h of fermentation and 174 metabolites were present in all 4 fermentations (Supplemental Figure 1). Fermentations that were inoculated with kefir grains contained 9 unique compounds (5 with positive IDs), while the three kefir consortia included 2 (kefir consortium, 2 positive IDs), 3 (kefir consortium without yeasts, 1 positive ID), and 2 (kefir consortium without lactobacilli, 0 positive IDs) unique compounds. The unique compounds identified for each fermentation are included in Supplemental Table 1.A total of 51 compounds differed between the fermentations with kefir grains and kefir consortia after 18 h of fermentation (Figure 2A). In particular, the relative abundance of many esters such as ethyl acetate, as well as ethanol, was higher in fermentations started with kefir grains. Conversely, the concentrations of organic acids and aldehydes including butanoic acid, acetic acid, and benzaldehyde were higher in fermentations with reconstituted kefir consortia (Supplemental Figure 2A).

A total of 16 compounds differed significantly between the kefir consortium and the same consortium without lactobacilli (Figure 2B). Of these 16 compounds, 11 were present at higher levels in kefir made with the complete consortium, with organic acids especially being increased. Meanwhile, the absence of lactobacilli from the consortium corresponded to increased levels of 3-penten-2-one (E) (Supplemental Figure 2B). In contrast to the other 18 h comparisons, only 4 compounds were different ($P<0.05$) between kefir fermented with the complete consortium and that produced by a consortium that lacked yeasts, with D-limonene, 2-heptanone, and nonanoic acid being increased in kefirs made with the complete consortium, suggesting a role for *L. kefiri* and/or *L. kefiranofaciens* in their production (Figure 2C and Supplemental Figure 2C).

The differences in fermentations were further analyzed by comparing the intensity levels of the major organic acids, esters, and ethanol across all four fermentation types. For each of butanoic, hexanoic, and octanoic acid, the kefir consortium and consortium without yeasts had higher levels than the consortium without lactobacilli and kefir grain fermentations (Figure 3). Analysis of the esters showed even more marked differences than the organic acids. Of the 4 esters analyzed, only butanoic acid ethyl ester was identified in the three kefir consortium fermentations, with the highest levels being present in kefir consortium without yeast and the complete kefir consortium, followed by the without lactobacilli and finally the grain fermentations (Figure 4 consortium). Conversely, the grain fermented kefir was the only kefir with detectable levels of each of 1-butanol-3-methyl-acetate, ethyl acetate, and propanoic acid 2-hydroxy-ethyl ester (Figure 4). In addition, ethanol levels were lower in kefirs produced with the consortium without lactobacilli fermentation than all other fermentations (Figure 5).

3.4 HPLC analysis of microbial metabolites

HPLC analysis of sugars and organic acids also showed differences between kefir types after 18h of fermentation. Both glucose and galactose were lower in kefir made with the kefir consortium without lactobacilli fermentations than in each of the other fermentation types, while traditional kefir also exhibited higher levels of glucose than the complete kefir consortium fermentation (Figure 5). Acetic acid levels in kefir fermented with the consortium without yeasts were higher than all three of the other kefir fermentations (Figure 5), with the complete consortium being higher than traditional kefir and both complete consortium and traditional kefir being higher than the consortium without lactobacilli. Lactic acid was higher in both the complete consortium and consortium without yeasts than in traditional kefir and kefir fermented with the lactobacilli-free consortium (Figure 5).

3.5 Metabolite Levels Change over the Course of Fermentation

In order to determine how the metabolite profile of the different kefirs progressed over time, we compared fermentations after both 12 and 18 h. Traditional kefir increased in multiple ethyl esters, as well as the organic acids hexanoic and acetic acid after 18 h of fermentation. The only compound with significantly higher levels in the 12 h traditional kefir samples was heptane, 4-methyl- (supplemental figure 3A). There was a much larger number of compounds that were different between 12 and 18 h of fermentation in the complete consortium kefir than in traditional kefir. The concentrations of butanoic, n-decanoic, nonanoic, acetic, hexanoic, and octanoic acids as well as the concentration of various esters were higher in kefir made with the complete consortium after 18 h (Supplemental Figure 3B). Both the consortium without lactobacilli and consortium without yeast fermentations closely resembled the complete consortium kefir in the changes observed between the 12 and 18 h time points with increases in a number of organic acids

in the 18 h samples along with increases in a small number of esters (Supplemental Figure 3C and D).

Results from HPLC also indicated changes in metabolite composition between 12 and 18 h of fermentation. There were differences observed across all metabolites and all fermentation types except with respect to levels of galactose with the lactobacilli free consortium kefir and ethanol with the complete consortium kefir (Supplemental Figure 4 A-E).

4. Discussion

This study examined metabolic differences between kefir produced with a traditional grain and a reconstituted kefir consortium fermentation, as well as how alteration of the kefir consortium impacted metabolite production during fermentation. We achieved this using GC×GC-TOFMS and HPLC to analyze volatile compound profiles, organic acids, and simple sugars at both 12 and 18 hours of fermentation. This is the first study to compare metabolite profiles in a traditional kefir fermentation to a reconstituted consortium that is made up of the core microbes present in the grain fermentation. This study is also the first, to our knowledge, to compare multiple kefir fermentations with demonstrated differences in health benefits in animal trials.

Interestingly, the reconstituted kefir consortium and the traditional kefir differed significantly in metabolite profile. Particularly, multiple esters, glucose, and ethanol were higher in traditional kefir when compared to the complete kefir consortium, while the kefir consortium fermentation contained more organic acids and aldehydes. The increased levels of esters in the traditional kefir, and particularly the fact that three esters were present only in the traditional kefir, along with higher levels of ethanol, may indicate an increased role of yeast metabolism in traditional kefir (Abeijón Mukdsi et al., 2009; Dragone et al., 2009; Liu et al., 1998; Longo & Sanromán, 2006; Sumby et al., 2010; Walsh et al., 2016). Indeed, Walsh *et al.* found that *Saccharomyces cerevisiae* was

strongly correlated with levels of both esters and ethanol in kefir fermentations (Walsh et al., 2016). Conversely, the higher levels of organic acids and aldehydes in the reconstituted kefir consortium fermentation indicates that these fermentations are dominated by lactic acid bacterial metabolism and potentially lactobacilli specifically, as *L. kefiranofaciens* has been associated with proteolysis in kefir fermentations A decreased role of yeast metabolism and subsequent increase in the role of lactobacilli in the reconstituted kefir consortium in comparison to traditional kefir explains the increase in levels of organic acids and aldehydes present in the complete consortium. Lactic acid is a major metabolite of carbohydrate metabolism by lactobacilli; heterofermentative lactobacilli additionally produce acetic acid or ethanol as main metabolites of sugar metabolism (Gänzle, 2015; Leroy & De Vuyst, 2004; Urbach, 1995; Walsh et al., 2016; Zalán et al., 2010). Aliphatic fatty acids with 4 or more carbons are released from milk fat by esterases (McSweeney et al., 2020); lactic acid bacteria do not produce these fatty acids or produce only trace amounts as product of amino acid metabolism. Additionally, lactic acid bacteria in dairy fermentations have been associated with the formation of multiple aldehydes, such as benzaldehyde, benzeneacetaldehyde, and 3-methylbutanal, metabolites of amino acids (Smit et al., 2005; Stefanovic et al., 2017; Van Kranenburg et al., 2002). Alongside past work that shows that *L. kefiranofaciens* plays a key role in proteolysis during kefir fermentation, these findings strongly suggest an increased role of lactobacilli in the reconstituted kefir consortia (Blasche et al., 2021; Cui et al., 2023).

This apparent shift towards a more lactic acid bacteria, and seemingly specifically lactobacilli, dominant fermentation may be due to differences in the starting ratios of bacteria and yeast between the kefir consortium and traditional kefir, as traditional kefir grains do not contain an equal mix of all organisms present (Dobson et al., 2011; Marsh et al., 2013). The glucose and

galactose concentrations indicate that lactobacilli are main contributors to β-galactosidase activity. Most kefir yeasts do not express β-galactosidases and *Lc. lactis* uses phospho-β-galactosidase, which does not result in accumulation of glucose or galactose (Gänzle & Follador, 2012). Lactose hydrolysis by lactobacilli makes the substrate available for other microbes in kefir communities that do not express β-galactosidases (Blasche et al., 2021).

The spatial arrangement of microorganisms within the kefir grain may also play a role in the fermentation dynamics, as different microbes are found in different areas of the grain (Lu et al., 2014; Marshall et al., 1984; Toba et al., 1990). While the composition of the kefir grain remains relatively unchanged during fermentation, the liquid portion is colonized sequentially by members of the kefir consortium. Lactose hydrolysis and other metabolic activities of pioneering microbes makes the substrate accessible for subsequent colonization by others (Blasche et al., 2021). These differences between the reconstituted kefir consortium and traditional kefir are interesting as, although they do not seem to alter the impact of the kefir on host cholesterol and lipid metabolism, they may prove to be important to the sensory characteristics of the finished product. Esters such as ethyl acetate and 1-butanol-3-methyl-acetate are generally associated with fruity aromas such as banana, pineapple, or apple, while organic fatty acids such as octanoic or acetic acid produce aromas described as cheesy or vinegar-like, and aldehydes can range in aroma from malty (3-methylbutanal) to almond or cherry (benzaldehyde) (Blaya et al., 2018; Walsh et al., 2016). These possible sensory differences should be further examined using a sensory analysis, as the perceived flavours and aromas of a product are the result of complex interactions between compounds present in the product (Cayot, 2007; Heiniö et al., 2003; Pan et al., 2014; Rapp, 1998; Viljanen et al., 2014).

Comparison of the complete consortium kefir to kefir fermented with lactobacilli-free consortium revealed higher levels of several organic acids in the complete consortium fermented kefir, while there were very few compounds that were different when the yeasts were removed. This increase in several organic acids in the complete kefir consortium when compared to the lactobacilli free kefir at 18 h, and the relatively minor differences observed between the complete consortium and yeast free kefir further point to the lactobacilli population being a major contributor to organic acid production and lipolysis during fermentation. Lactobacilli are capable of producing butanoic acid in addition to lactic and acetic acids but butanoic acid in fermented dairy products is more likely to originate from lipolysis (Gänzle, 2015; Zalán et al., 2010). The observed differences between the complete kefir consortium and the no lactobacilli and no yeast variations showed only nonanoic acid was similarly increased in the complete kefir consortium when compared to both the consortium variants, indicating that both lactobacilli and yeast contribute to the production of this organic acid. While nonanoic acid has not been associated with any cholesterol lowering effects, it has been shown to have mild anti-inflammatory potential due to cyclooxygenase (COX)-1 inhibitory activity (Henry et al., 2002). Other fatty acids, such as butyrate and acetate, have shown potential to benefit host health (Xiong et al., 2022), indicating that nonanoic acid may be a compound of interest for future studies into kefir's health promoting effects.

Characterization of changes to the volatile compound profile of traditional kefir over the course of fermentation revealed a trend towards an increase in ester levels from 12 to 18 h potentially indicating that alcohol and organic acid production is relatively constant throughout fermentation while esterification takes place later. As organic acids and alcohols are utilized by yeast in the formation of esters, this would explain why there was not more organic acids or alcohols present in the 12 h fermentation samples (Dzialo et al., 2017). Contrary to traditional kefir, for all 3

consortia fermented kefir the concentrations of a number of organic acids, including hexanoic and butanoic acid, phenylethyl alcohol, and a small number of large fatty acid esters increased between 12 and 18 h. This may further support the possibility of the kefir consortium fermentations being more bacterially, and specifically lactobacilli driven, as the increase in organic acids could indicate a lack of organic acid utilization for ester production by the yeast present in the fermentation.

In the current study, the GC×GC-TOFMS method allows for tentative identification of volatile compounds and relative quantification of compounds across samples. It would be appropriate in future studies to utilize certified standards for compounds of interest to allow for absolute quantification of relevant volatiles. While sensory characteristics of compounds of interest have been discussed, this study did not perform sensory analysis of the different kefir fermentations. In order to be certain of any impact of fermentation inoculum on kefir fermentation a sensory comparison of the products would have to be undertaken and should be considered for future studies. One family of metabolite not examined in this study was peptides, which have been shown to have beneficial effects in past animal trials (Malta et al., 2022; M. C. Tung et al., 2020; Y.-T. Tung et al., 2017). The recent advent of databases to better identify bioactive peptides present in food fermentations may also allow more streamlined and accurate ID of compounds of interest for future study (Gobbi et al., 2019).

This study found that, while measures such as pH and microbial loads are similar between traditional kefir and a reconstituted kefir consortium fermentation, kefir made with a reconstituted consortium of microbes had a significantly different metabolite profile than the traditional example. These findings indicate that despite the ability to replicate health benefits of traditional kefir, further sensory analysis to determine consumer acceptability may be necessary prior to the implementation of reconstituted kefir consortium as a starter culture for kefir production. Kefir is

made with pasteurized milk in Canada and does not traditionally contain pathogenic microbes, while the low pH achieved through acidification by lactic acid bacteria will generally inhibit any contaminating pathogenic organisms other than unwanted yeast or molds. The critical control point for kefir production in Canada is achieving a pH of 4.6 or lower by the end of fermentation as defined by the producer, which the kefir consortium fermentation achieved after 18 hours. Combined with the lack of any microbial standards for kefir in Canada, this makes the implementation of this consortium a strong possibility.

5. Conclusion

In conclusion, this study examined metabolite profiles of kefir products generated using grain fermentation or kefir consortia fermentations with varying microbial compositions. We were able to identify a distinct difference in metabolite profiles between traditional grain fermented kefir and a consortium fermented example, with the traditional kefir exhibiting increased ester production and the consortium kefir having higher levels of organic acids and aldehydes. Comparisons of the complete kefir consortium and lactobacilli free kefir consortium showed lower levels of organic acid production, while the volatile profiles were relatively unchanged between the complete kefir consortium fermentations and those with the yeast removed; potentially indicating an important role for the lactobacilli population in the fermentation profile of the reconstituted kefir consortium. This study represents a first examination of the importance of the kefir grain structure in fermentation dynamics, while also providing valuable insight into how different populations of organisms contribute to planktonic kefir fermentation. This is especially important given the potential for future studies utilizing multi-omic approaches to better understand these microbial interactions during fermentation. Future work should focus on sensory analysis of these different kefir fermentations, as well as analysis of gene expression profiles to examine how microbial

behaviour differs between traditional kefir and the reconstituted kefir consortium. We have also identified nonanoic acid as a metabolite of interest in conferring cholesterol lowering effects of kefir consumption.

Conflicts of Interest

BCTB, PDC and BPW hold a patent for the method used to produce the pitched kefir used in the trial. ND, RPD, SLN, APM, AJF, GG, JJH, and MG have no conflicts to declare.

Author Contributions

BCTB, JJH, PDC, and BPW designed research; BCTB, AJF, RPD, and GG conducted research; BCTB, ND, RPD, SLN, APM, and AJF analyzed data; BCTB, ND, RPD, APM, JJH, MG, PDC, and BPW wrote the paper; BPW had primary responsibility for final content. All authors have read and approved the final manuscript.

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Figure legends

Figure 1. PCA of volatile metabolites in kefir after fermentation for 12 and 18 h.

Figure 2. Volcano plot of significantly different compounds between traditional and complete consortium kefir (A), complete consortium and consortium without lactobacilli kefir (B), and complete consortium and consortium without yeast kefir (C) following 18 h of fermentation.

Figure 3. Boxplots showing intensity levels of butanoic acid (A), hexanoic acid (B), and octanoic acid (C) between traditional grain fermented kefir and three kefir consortia of varying microbial composition following 18 h of fermentation. Peak intensity was obtained using GC×GC-TOFMS. Means that do not share a letter are significantly different (P<.05). N=3.

Figure 4. Boxplots showing intensity levels of butanoic acid ethyl ester (A), 1-butanol-3-methyl-acetate (B), ethyl acetate (C), and propanoic acid 2-hydroxy-ethyl ester (D) between traditional grain fermented kefir and three kefir consortia of varying microbial composition following 18 h of fermentation. Peak intensity was obtained using GC×GC-TOFMS. Means that do not share a letter are significantly different (P<.05). N=3.

Figure 5. Boxplots showing intensity levels of Glucose, Galactose, Acetic Acid, Lactic Acid, and Ethanol between traditional grain fermented kefir and three kefir consortia of varying microbial composition following 18 h of fermentation as measured by HPLC. Means that do not share a letter are significantly different (P<.05). N=2-3.

Tables

Table 1. Microbial cell counts [Log(CFU/mL)] and pH of kefir fermented with kefir grains and reconstituted kefir consortia after 18 h of fermentation. Data are shown as means ± standard deviations of 5 or 6 biological repeats. Cells with different letters in the same column indicate statistically significant differences ($P < 0.05$)

Starter culture	Log CFU/mL Bacteria	Log CFU/mL Yeast	pH
Kefir grain	8.47± 0.11[a]	6.69± 0.15[a]	4.74± 0.20[a]
Kefir consortium	8.38± 0.07[a]	6.82± 0.13[a]	4.56± 0.19[a]
Kefir consortium without lactobacilli	8.32± 0.20[a]	6.83± 0.13[a]	4.62± 0.14[a]
Kefir consortium without yeasts	8.38± 0.08[a]	n.d.[b]	4.48± 0.13[a]

n.d. = not detected. Limit of detection = 100 cfu/ml

Figures

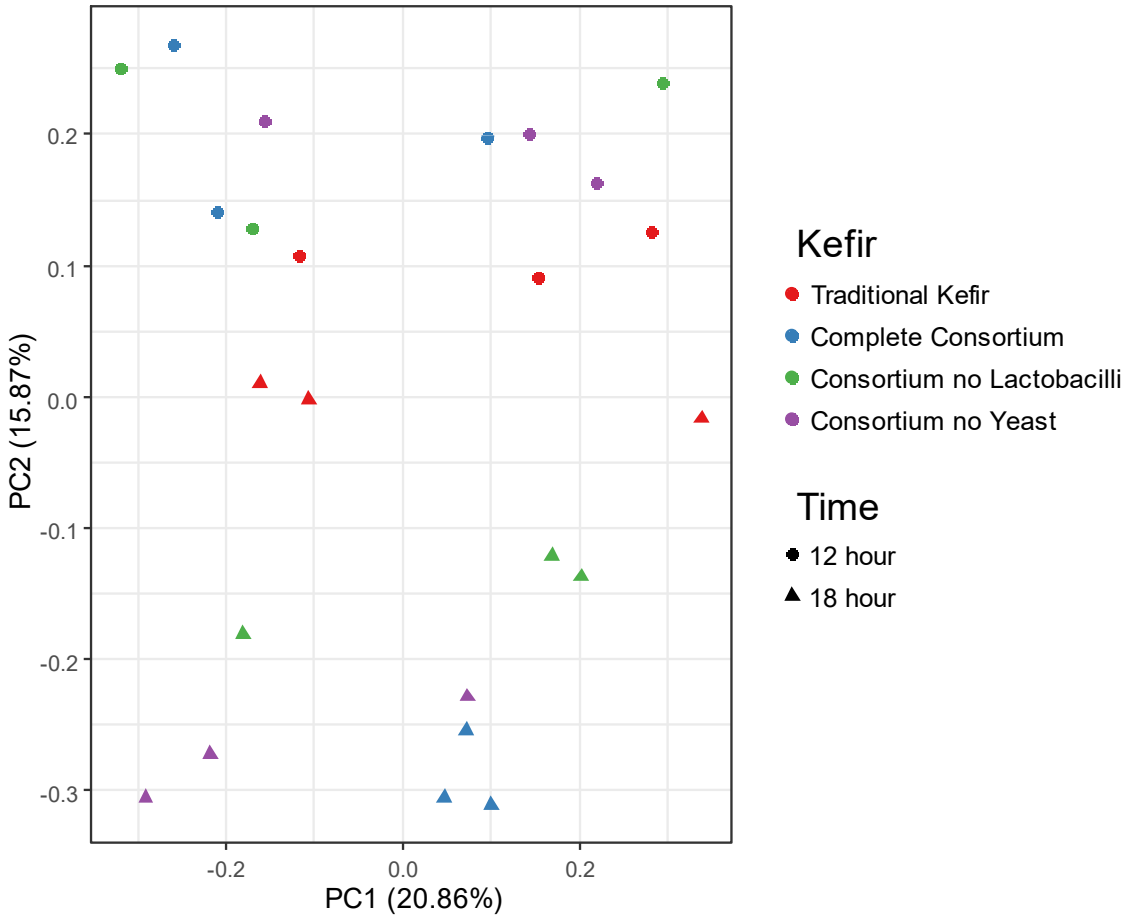


Figure 1

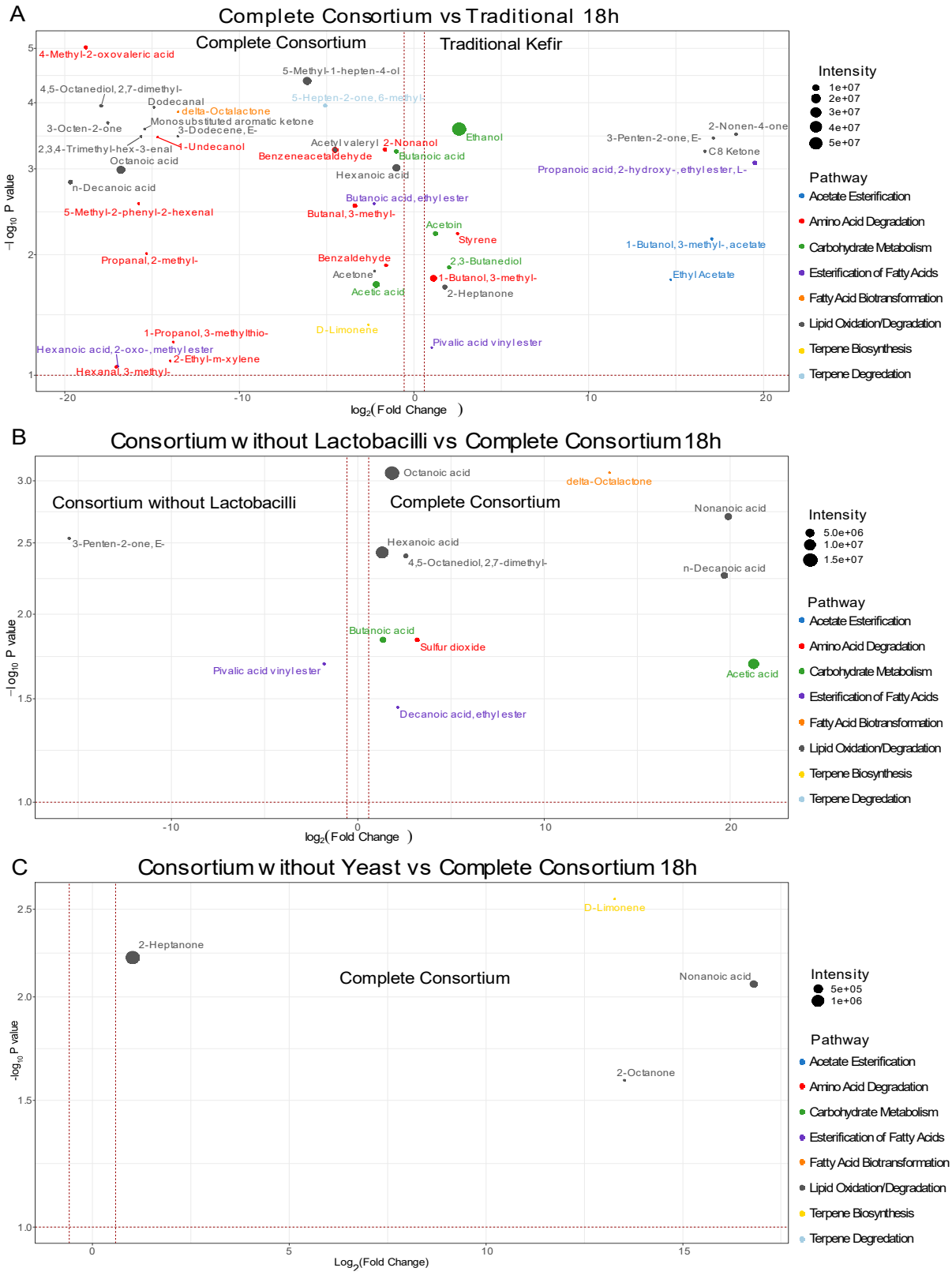
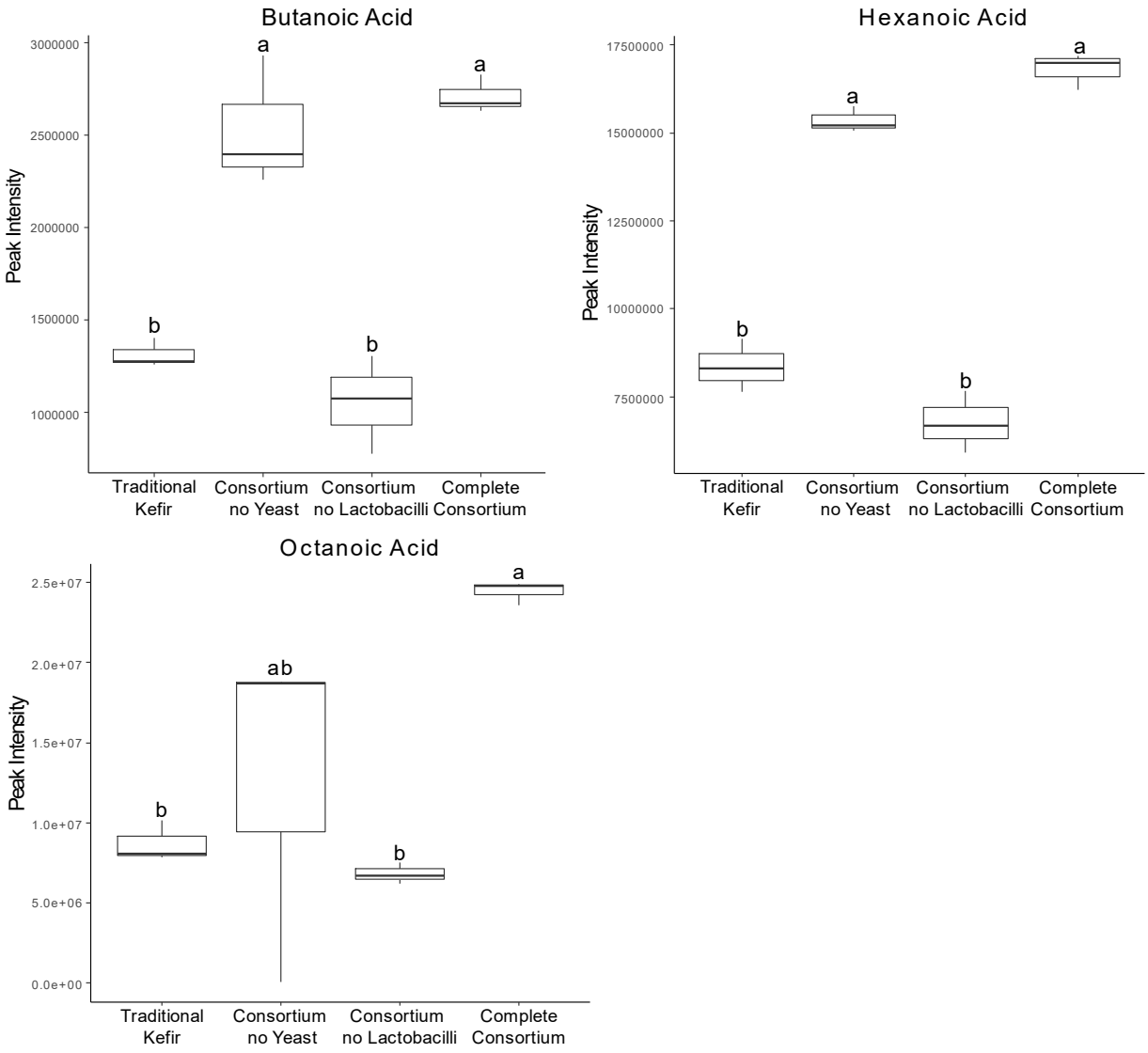

A
Complete Consortium vs Traditional 18h
Complete Consortium
Traditional Kefir
4-Methyl-2-oxovaleric acid
5-Methyl-1-hepten-4-ol
4,5-Octanediol, 2,7-dimethyl-
Dodecanal
5-Hepten-2-one, 6-methyl-
delta-Octalactone
Monosubstituted aromatic ketone
3-Octen-2-one
3-Dodecene, E-
2,3,4-Trimethyl-hex-3-enal
1-Undecanol
Octanoic acid
Acetyl valeryl
2-Nonanol
Benzeneacetaldehyde
Ethanol
Butanoic acid
Hexanoic acid
3-Penten-2-one, E-
2-Nonen-4-one
C8 Ketone
Propanoic acid, 2-hydroxy-, ethyl ester, L-
n-Decanoic acid
Butanoic acid, ethyl ester
5-Methyl-2-phenyl-2-hexenal
Butanal, 3-methyl-
Acetoin
Styrene
1-Butanol, 3-methyl-, acetate
Propanal, 2-methyl-
Benzaldehyde
2,3-Butanediol
Acetone
1-Butanol, 3-methyl-
Ethyl Acetate
Acetic acid
2-Heptanone
D-Limonene
1-Propanol, 3-methylthio-
Pivalic acid vinyl ester
Hexanoic acid, 2-oxo-, methyl ester
2-Ethyl-m-xylene
Hexanal, 3-methyl-
−log10 P value
log2(Fold Change)
Intensity
1e+07
2e+07
3e+07
4e+07
5e+07
Pathway
Acetate Esterification
Amino Acid Degradation
Carbohydrate Metabolism
Esterification of Fatty Acids
Fatty Acid Biotransformation
Lipid Oxidation/Degradation
Terpene Biosynthesis
Terpene Degredation
B
Consortium without Lactobacilli vs Complete Consortium 18h
Consortium without Lactobacilli
Complete Consortium
Octanoic acid
delta-Octalactone
Nonanoic acid
3-Penten-2-one, E-
Hexanoic acid
4,5-Octanediol, 2,7-dimethyl-
n-Decanoic acid
Butanoic acid
Sulfur dioxide
Pivalic acid vinyl ester
Acetic acid
Decanoic acid, ethyl ester
−log10 P value
log2(Fold Change)
Intensity
5.0e+06
1.0e+07
1.5e+07
Pathway
Acetate Esterification
Amino Acid Degradation
Carbohydrate Metabolism
Esterification of Fatty Acids
Fatty Acid Biotransformation
Lipid Oxidation/Degradation
Terpene Biosynthesis
Terpene Degredation
C
Consortium without Yeast vs Complete Consortium 18h
Complete Consortium
D-Limonene
2-Heptanone
Nonanoic acid
2-Octanone
-log10 P value
Log2(Fold Change)
Intensity
5e+05
1e+06
Pathway
Acetate Esterification
Amino Acid Degradation
Carbohydrate Metabolism
Esterification of Fatty Acids
Fatty Acid Biotransformation
Lipid Oxidation/Degradation
Terpene Biosynthesis
Terpene Degredation

Figure 2.



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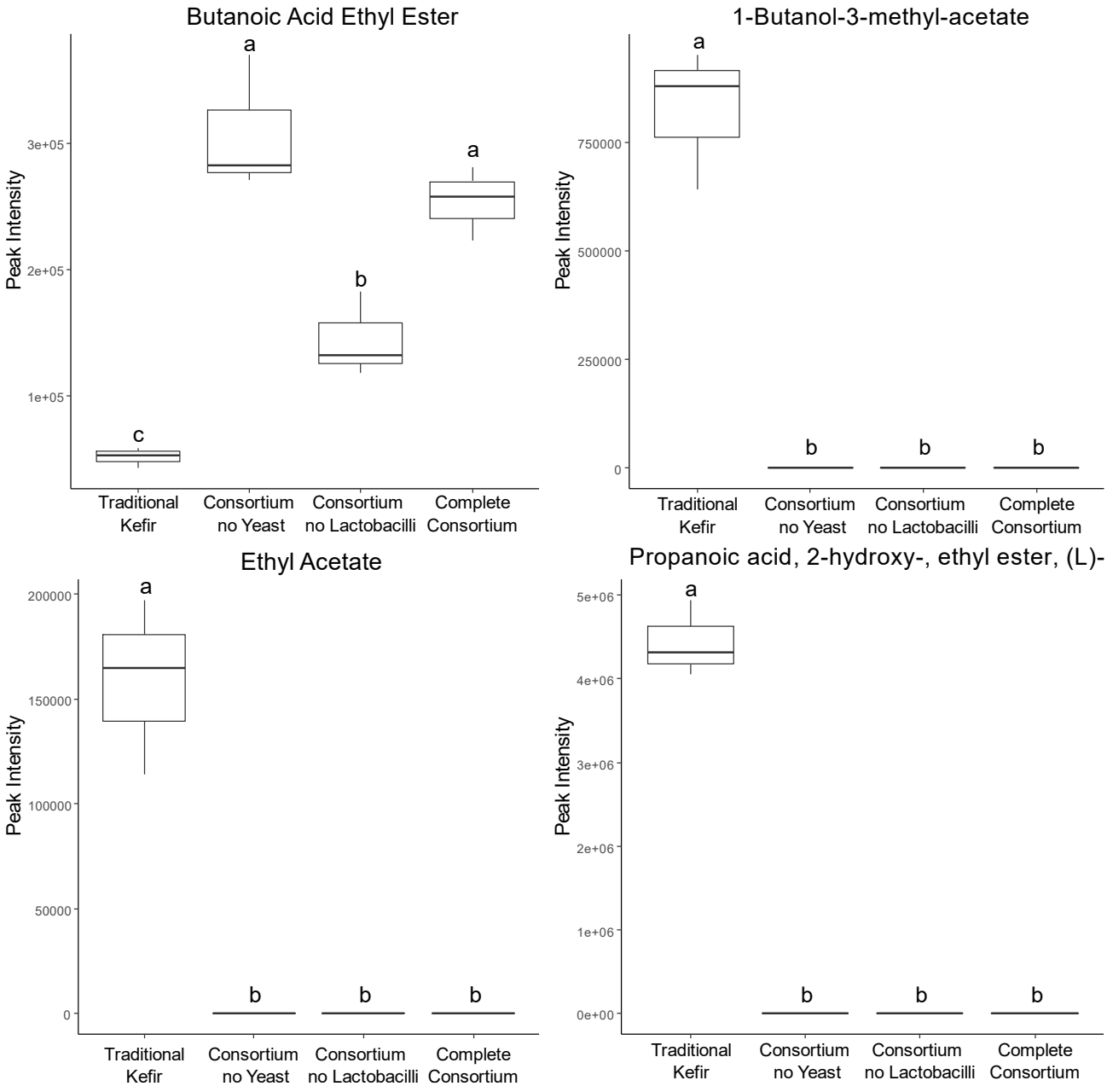

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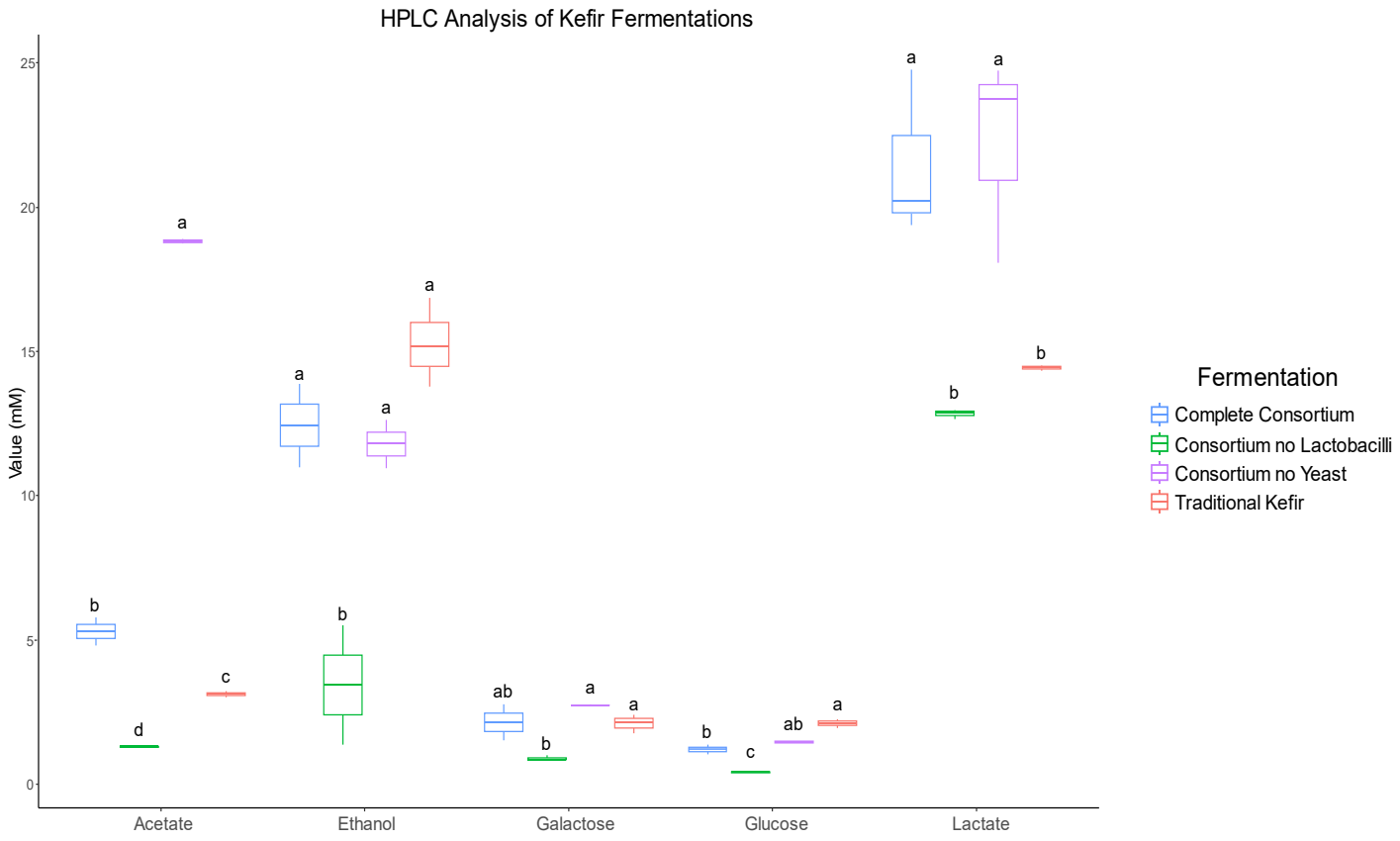
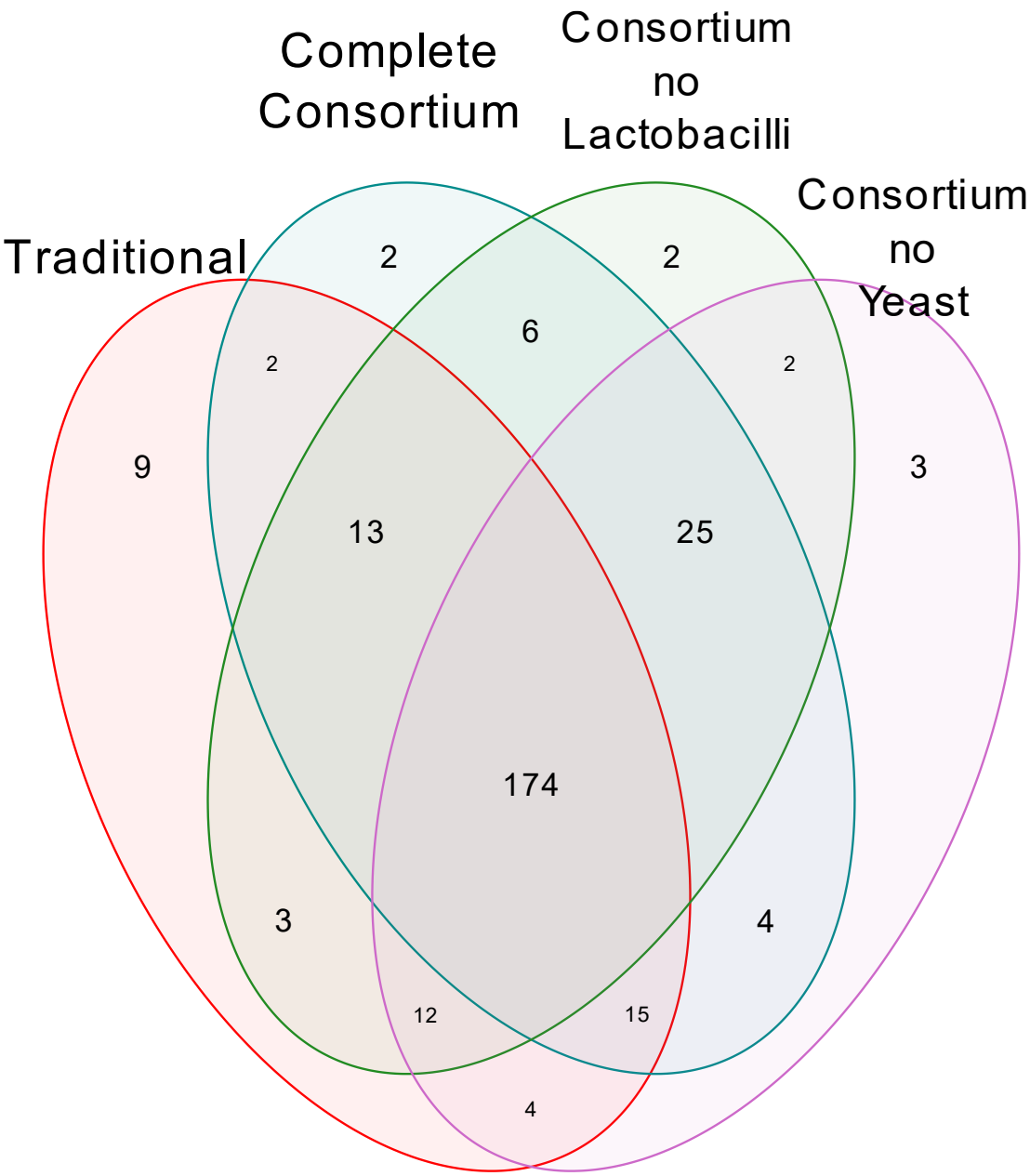


612

613 Figure 5.

614

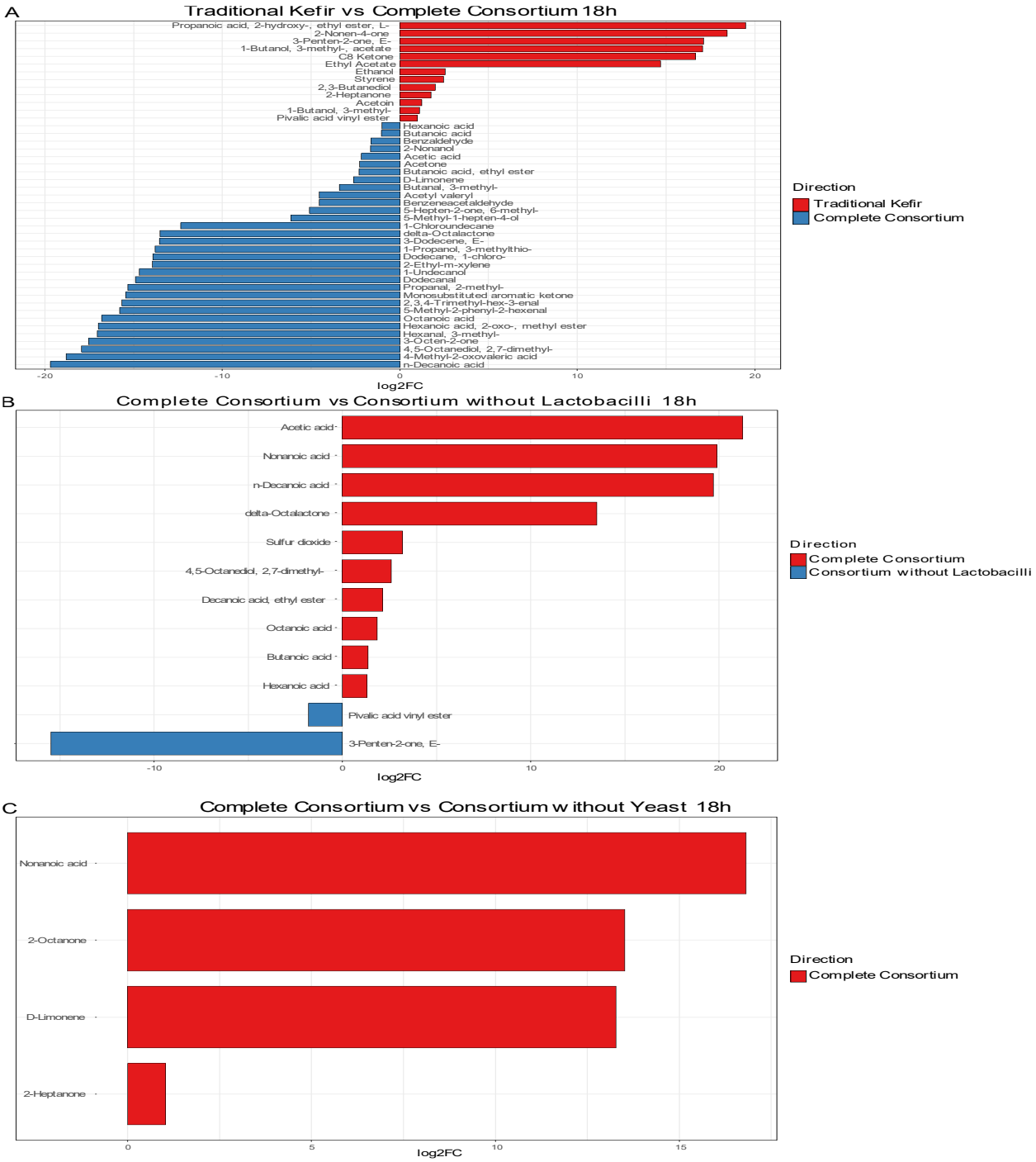
Supplemental Figures



Supplemental Figure 1. Venn diagram of the 4 kefir fermentations showing the number of shared and unique compounds for each possible comparison.

Starter culture	Unique Metabolites in GC×GC-TOFMS Analysis with Positive ID
Kefir grain	Ethyl Acetate Propanoic acid, 2-hydroxy-, ethyl ester, (L)- 1-Butanol, 3-methyl-, acetate C8 Ketone 2-Nonen-4-one
Kefir consortium	Tetrahydrogeranyl formate Dodecane, 2,7,10-trimethyl-
Kefir consortium without lactobacilli	No Compounds with ID
Kefir consortium without yeasts	Ethyl 4-ethoxybenzoate

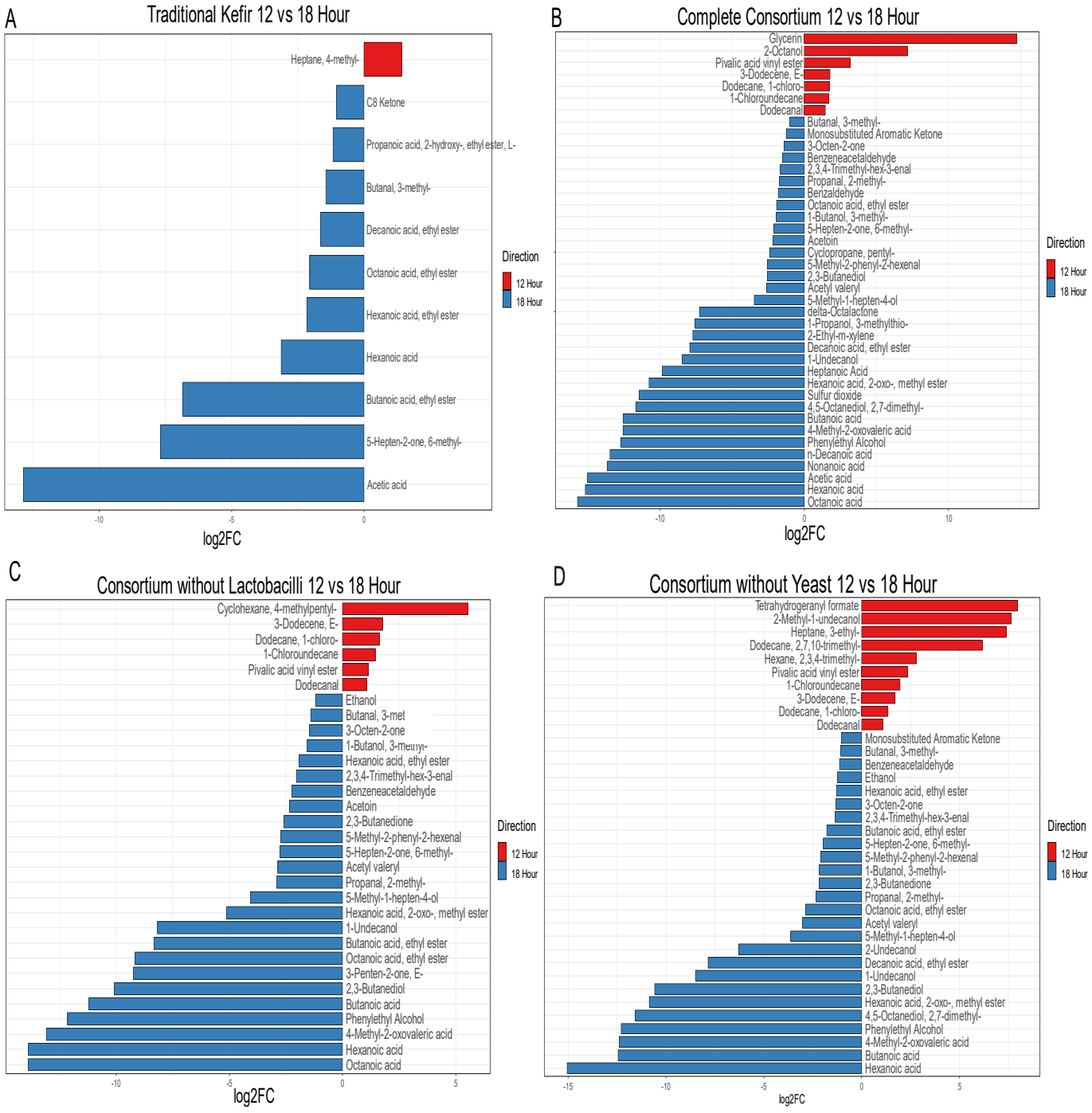
Supplemental Table 1. Compounds uniquely identified in each fermentation with a positive ID.



622

623 Supplemental Figure 2. LDA plots of significantly different compounds between traditional and
624 complete consortium kefir (A), complete consortium and consortium without lactobacilli kefir (B),
625 and complete consortium and consortium without yeast kefir (C) following 18 h of fermentation.

626



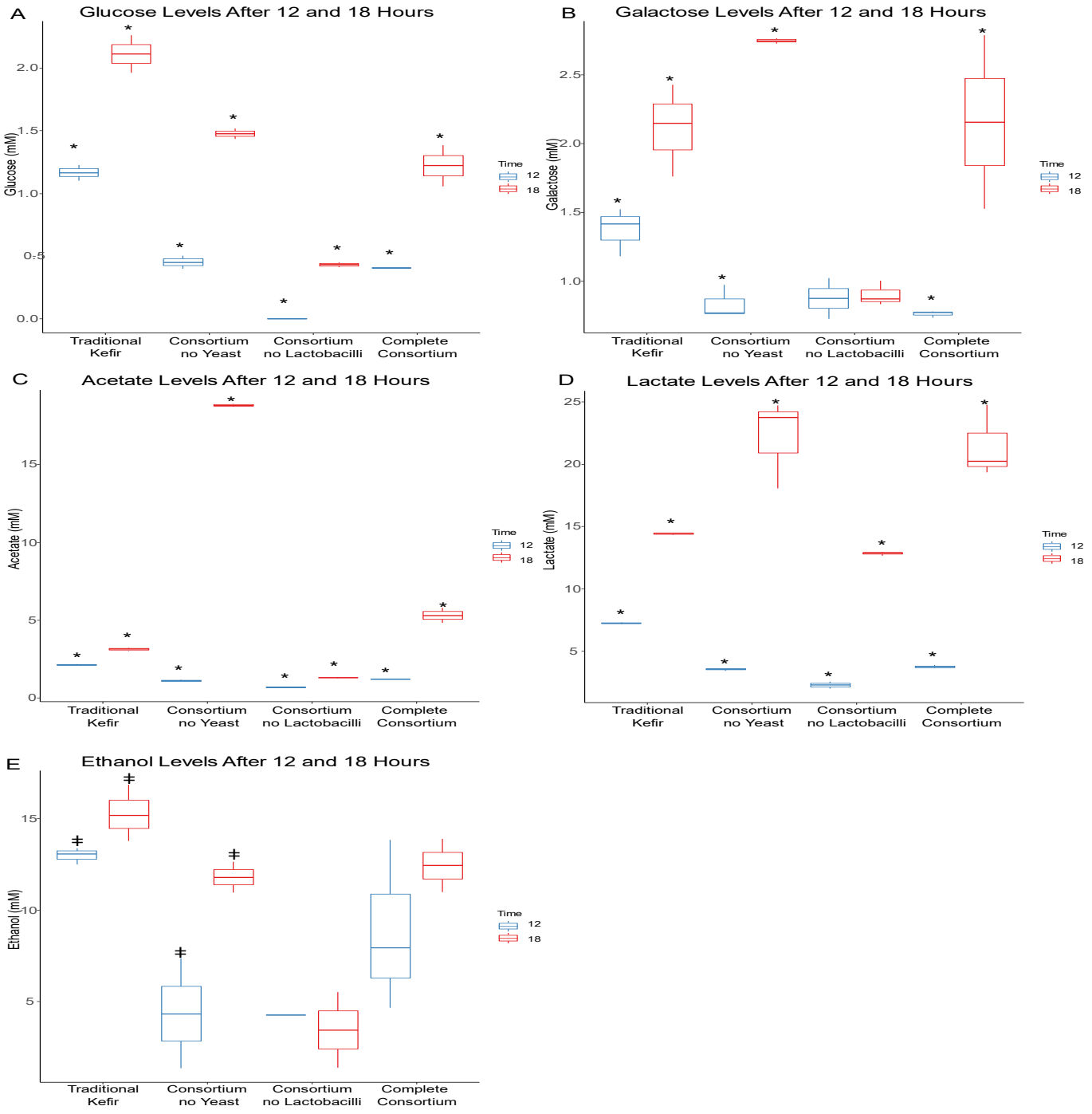
627

628 Supplemental figure 3. Lefse plot of compounds with significantly different levels between 12 and

629 18 h of fermentation in traditional kefir (A), complete kefir consortium (B), consortium without

630 lactobacilli (C), and consortium without yeast (D) kefir.

631 .



632

633 Supplemental figure 4. Boxplots of compounds analyzed by HPLC in kefir after both 12 and 18 h

634 of fermentation. * indicates a significant difference between 12 and 18 h measurements within a

635 single group ($P<0.05$), while ǂ indicates a trend for significance ($P<0.10$). N=2-3.