ELECTRONIC SUPPLEMENTARY INFORMATION (E.S.I.)

On the Dynamics of Kefir Volatome

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Experimental section (extended)

Analysis by matrix-assisted laser desorption/ionisation mass spectrometry

Owing to its potential in metabolomics research (J. L. Edwards and R. T. Kennedy, *Anal. Chem.*, 2005, **77**, 2201; A. Amantonico *et al.*, *Anal. Chem.* 2010, **82**, 7394; J.-B. Hu *et al.*, *Anal. Chem.*, 2012, **84**, 5110.; A. J. Ibañez *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 2013, **110**, 8790.), MALDI-MS was implemented in semi-quantitative analysis of intracellular metabolites of *S. cerevisiae* cells. A 1-mL aliquot of yeast culture in YM broth was obtained from the 20-mL GC vial, and transferred into a 2-mL microcentrifuge tube held on ice. Subsequently, 1 mL of ice-cold water was added to slow down metabolic processes in cells. The resulting suspension of cells was centrifuged at 3,300 *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in 2 mL of ice-cold water, followed by centrifugation (3,300 *g*, 5 min). This operation was repeated. Following the three-step washing procedure, the pellet was resuspended in 400 μL of ice-cold water, and 50 mg of acid-washed glass beads were added to facilitate disruption of yeast cells. The suspension was vortex-mixed 3-5 times for 30 s. The cells were kept on ice for 30 s between the vortexing cycles. Subsequently, the glass beads and cell debris were removed by centrifuging the mixture 5 times for 1 min at 14,100 *g*. Also in this case, the cells were kept on ice for 1 min between the cycles. The resulting cell extract was stored at -20 °C until analysis. Right before the analysis, a 0.5-μL aliquot of the cell extract was deposited on the MTP 384 massive MALDI target (Bruker Daltonics, Bremen, Germany). Then, 0.5 μL of 10^{-6} M $^{13}C_{10}$-ATP standard in water, and 0.5 μL of 9 mg mL^{-1} 9-aminoacridine matrix in acetone, were added. After drying at room temperature, the resulting sample was analysed by MALDI-MS.

The MALDI-MS instrument (Autoflex III Smartbeam; Bruker Daltonics) is equipped with solid-state laser (λ = 355 nm). In this study, medium laser spot size (60-70 μm) was selected. The instrument was operated in the negative-ion mode. The *m/z* range used in the analysis was 200-1000 u e^{-1} with the cut-off value set to 200 u e^{-1}. Every final mass spectrum was an average of subspectra obtained during 200 laser shots fired with a frequency of 50 Hz.

Analysis by liquid chromatography coupled to mass spectrometry

A 0.5-mL aliquot of fresh kefir was transferred into a 2-mL microcentrifuge tube, and mixed with 1 mL of cold methanol. The mixture was vortexed for 5 min, then centrifuged at 14,100 *g* for 10 min. The supernatant of kefir extract was filtered using polyterafluorothylene (PTFE) syringe filters with 0.2-μm pores (Pall Corporation, Ann Arbor, MI, USA), then stored at -20 °C until analysis.

An ultra-high performance liquid chromatography system (Dionex UltiMate 3000; Thermo Fisher Scientific, Waltham, MA, USA), coupled with an ion-trap mass spectrometer (Amazon Speed; Bruker Daltonics), equipped with an electrospray ion (ESI), source was used to carry out pilot analysis of compounds extracted from kefir (including the non-volatile ones). The C-18 Hypersil GOLD Silica column (length: 150 mm; diameter: 1 mm; particle diameter: 3 μm) was used for the separation. The column temperature was set to 30 °C. The flow rate of mobile phase was set to 50 μL min^{-1}. The mobile phase was composed of water (A) and methanol (B). The gradient elution program was: 0-5 min, 10% B; 5-25 min, 10-80% B; 25-30 min, 80% B.

Results and discussion (extended)

Analysis by matrix-assisted laser desorption/ionisation mass spectrometry

The similarity of secondary metabolic profiles obtained for kefir and single-species yeast cultures (**Figs. 2D**, **2F**, **3**, **S4-S6**) might support the hypothesis that yeast culture can – at least to some extent – be used as a simple metabolic model for kefir metabolism (after production). While the analytical methodology used here (HS-SPME-GC-MS) coped with the determination of volatile extracellular metabolites in kefir, it failed to provide any data on primary metabolites, such as nucleotides. Those compounds possess negative charge, and do not normally diffuse out of the cell. Therefore, we implemented another method, based on MALDI-MS, to assay those intracellular metabolites. Due to the difficulty to separate individual microbial species of kefir from the sample matrix, we could only perform such study on yeast cells obtained from the single-species yeast cell culture.

Adenine energy charge (EC) is an established parameter for evaluation of the energy state of cells at specific physiological conditions, and an important indicator of cellular

metabolic activity (Chapman *et al.*, *J. Bacteriol.*, 1971, **108**, 1072). EC is defined as (ATP+0.5ADP)/(ATP+ADP+AMP) (Atkinson, *Biochemistry*, 1968, **7**, 4030). On the basis of this equation, an EC of 1 may indicate the intracellular AMP is completely converted to ATP. On the other hand, an EC of 0 would indicate that intracellular ATP is completely converted to AMP. In general, growing cells have an EC within the range of 0.80-0.95. Low EC values (< 0.5) indicate cell death (Chapman *et al.*, *J. Bacteriol.*, 1971, **108**, 1072). **Fig. S7** shows the apparent energy change and relative abundance of selected primary metabolites, estimated from MALDI-MS analysis of *S. cerevisiae* liquid culture monitored over 72 h. Interestingly, the EC values did not change significantly, and high values were maintained over the period of 72 h (**Fig. S7A**). Since it was not possible to estimate the energy charges for yeast cells found in probiotic drinks, and because the nutrient medium of yeast culture does not faithfully emulate the nutrient composition of kefir, these values can only serve as an indirect indicator of the metabolic activities of probiotic yeast in the incubated kefir sample. Please note that the apparent energy charge was calculated based on the peak intensity corresponding to ATP, ADP, AMP and obtained from MALDI-MS experiment. Considering the occurrence of in-source decay, we used $^{13}C_{10}$-ATP to determine the degree of in-source decay, and correct the peak intensities before using them for calculations. The resulting values are referred to as the apparent energy charge because of the limited quantitative capabilities of the MALDI-MS method, and the anticipated differences in ionisation yields of AMP, ADP and ATP.

Apart from the adenosine phosphates, several other intracellular metabolites could be recorded in the MALDI mass spectra of *S. cerevisiae*, including: uridine diphosphate (UDP), uridine triphosphate (UTP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), uridine diphosphate glucose (UDP-Glc), as well as uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc). By calculating the ratio of the peak intensities corresponding to various intracellular metabolites, and the peak intensity corresponding to the internal standard ($^{13}C_{10}$-ATP), one can observe changes in the primary metabolism in *S. cerevisiae* in a culture medium, in which glucose is depleted during the initial stage of incubation (~ 24 h). The signal intensities of UDP-Glc (**Fig. S7B**), UTP (**Fig. S7C**), and GTP (**Fig. S7D**) normalised to the internal standard ($^{13}C_{10}$-ATP) are consistently lower in the data points obtained for the later stage of incubation, which is consistent with the depletion of glucose from the culture medium (**Fig. 4A**). The concentrations of some

intracellular metabolites rapidly change upon environmental stress exerted on the cells (for example, osmotic stress due to the elevated concentrations of ethanol). In fact, these metabolites are known to be sensitive to the limiting nutrient – glucose (Parrou *et al.*, *Microbiology*, 1997, **143**, 1891; Gasch *et al.*, *Mol. Biol. Cell*, 2000, 4241; Brauer *et al.*, *Mol. Biol. Cell*, 2008, **19**, 352; Boer *et al.*, *Mol. Biol. Cell*, 2010, **21**, 198). This effect – along with the depletion of glucose, and the occurrence of diauxic shift – may further influence the levels of various primary metabolites involved in the downstream primary metabolism (including UDP-Glc, UTP, and GTP).

Analysis by liquid chromatography coupled to mass spectrometry

We also carried out a pilot study in which we analysed non-volatile methanol-extractable substances present in kefir. Liquid chromatography (LC) with a reverse-phase C-18 column, connected to ion trap mass spectrometer via electrospray ion source, was used to separate non-volatile compounds present in kefir at different time points. Kefir samples were incubated at 28 °C, and aliquots were analysed every few hours during the period 0-72 h by LC-ESI-MS and LC-ESI-MS/MS. In this preliminary experiment, we could separate few compounds present in kefir, including those represented by the signals at the *m/z* 261.0 u e^{-1} (retention time: 3.4 min), 437.4 u e^{-1} (retention time: 8.3 min) and 603.9 u e^{-1} (retention time: 13.6 min). Interestingly, the signals at the *m/z* 261.0 and 437.4 u e^{-1} increased over time, but the signal at the *m/z* 603.9 u e^{-1} increased in the beginning of incubation but later decreased (**Fig. S8**). These preliminary results suggest that chemical dynamics can be observed in kefir within relatively short periods of time – also with respect to non-volatile compounds. Further studies are warranted to identify and quantify non-volatile metabolites which contribute to the chemical dynamics of kefir.

Additional table

Table S1. The LODs were calculated using the *S/N* = 3 criterion, where *S* is the peak amplitude and *N* is the root mean square (RMS) noise of baseline. Sample matrix: YM broth.

Compound*	*m/z* (u e^{-1})	Retention time	Calibration curve	R^2	Linear range (mg L^{-1})	LODs (μg L^{-1})
I. A.	43 ± 0.5	4.6	$y = 1.09\times10^6 x + 3.34\times10^5$	0.9988	0.05-20.00	22
E. H.	88 ± 0.5	5.8	$y = 3.88\times10^5 x + 3.55\times10^4$	0.9995	0.02-20.00	1
E. O.	88 ± 0.5	7.4	$y = 1.63\times10^5 x - 1.65\times10^4$	0.9991	0.02-20.00	4
P. A.	104 ± 0.5	8.0	$y = 2.70\times10^5 x - 3.17\times10^4$	0.9996	0.02-20.00	3
E. D.	88 ± 0.5	8.8	$y = 5.19\times10^4 x - 3.50\times10^4$	0.9787	0.05-20.00	43
Thymol	135 ± 0.5	8.2	$y = 1.21\times10^5 x - 3.10\times10^4$	0.9989	0.02-20.00	4

* I. A., isopentyl acetate; E. H., ethyl hexanoate; E. O., ethyl octanoate; P. A., phenethyl acetate; E. D., ethyl decanoate.

Additional figures

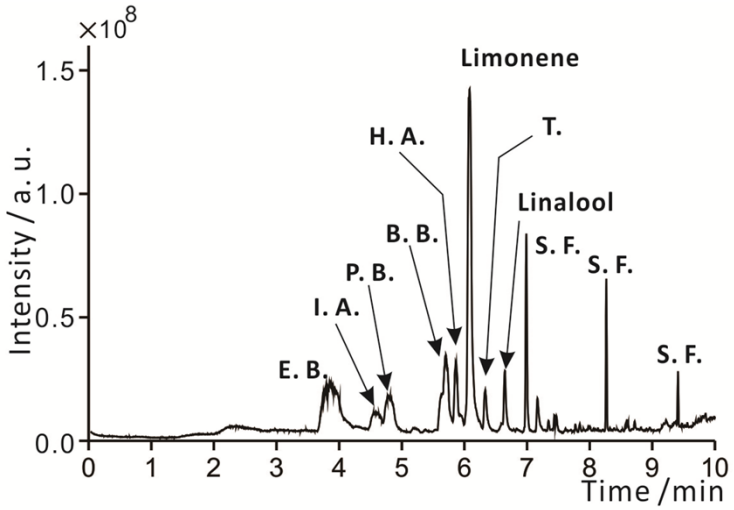

Figure S1. Result of HS-SPME/GC-MS analysis of a yogurt sample following 72-h incubation at 28 °C. PTIC: 80-130 u e^{-1}. Peaks: E. B., ethyl butyrate; I. A., isopentyl acetate; P. B., propyl butyrate; B. B., butyl butyrate; H. A., hexyl acetate; G. T., terpinene; S. F., contaminant related to the SPME fibre.

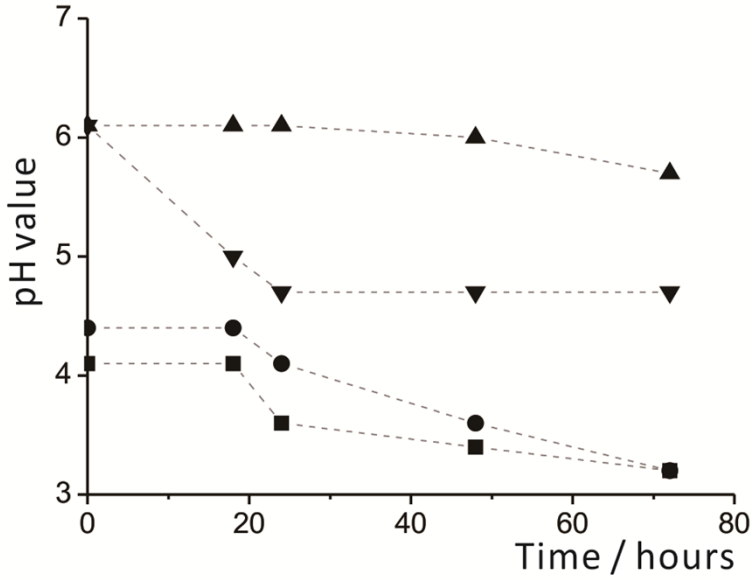

Figure S2. Acidity of (▲) YM broth medium, (▼) yeast grown in YM broth medium, (●) kefir, and (■) yogurt during incubation at 28 °C. Average values for two experimental replicates are shown.

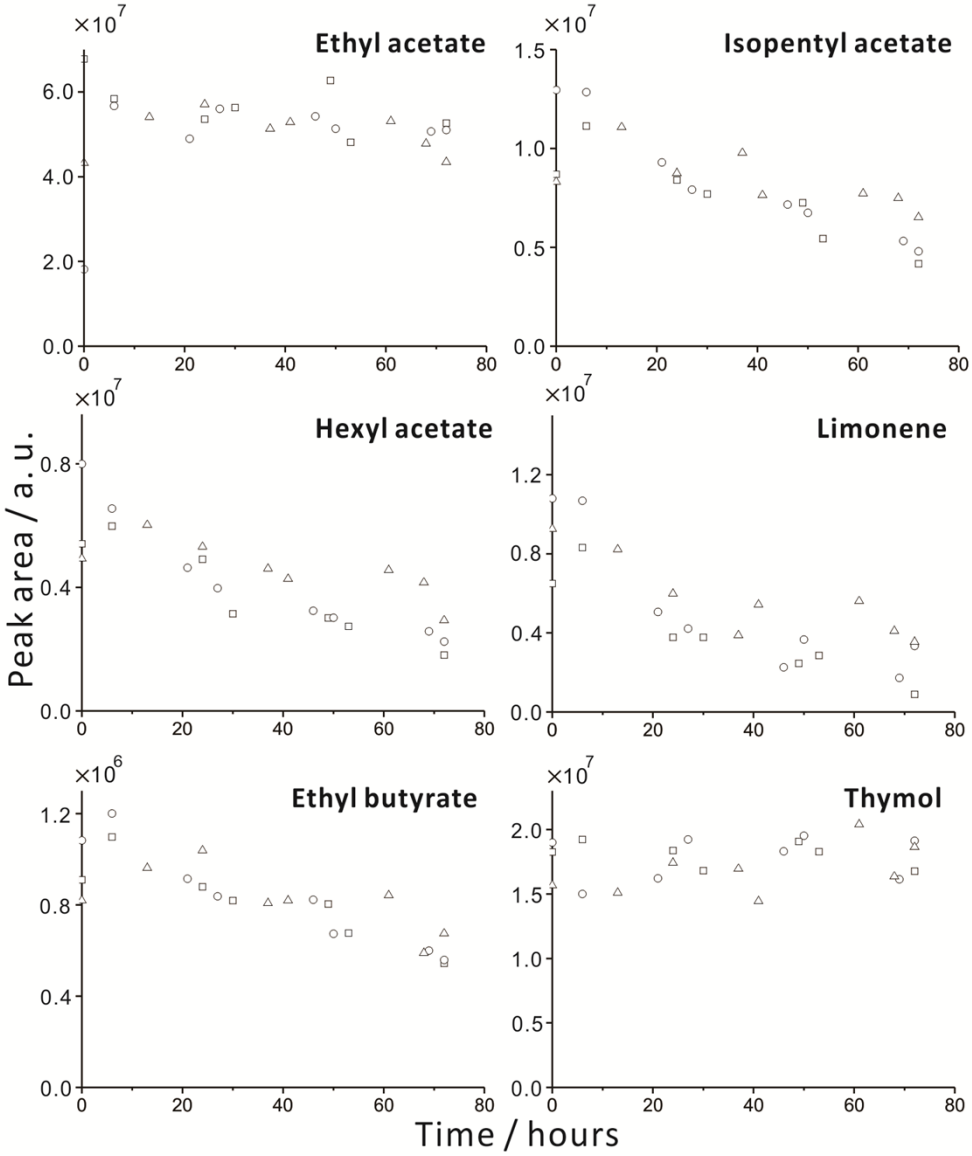

Figure S3. Dynamic monitoring of a yogurt sample by HS-SPME/GC-MS. Incubation temperature: 28 °C. The three symbols (square, circle, and triangle) indicate three experimental replicates.

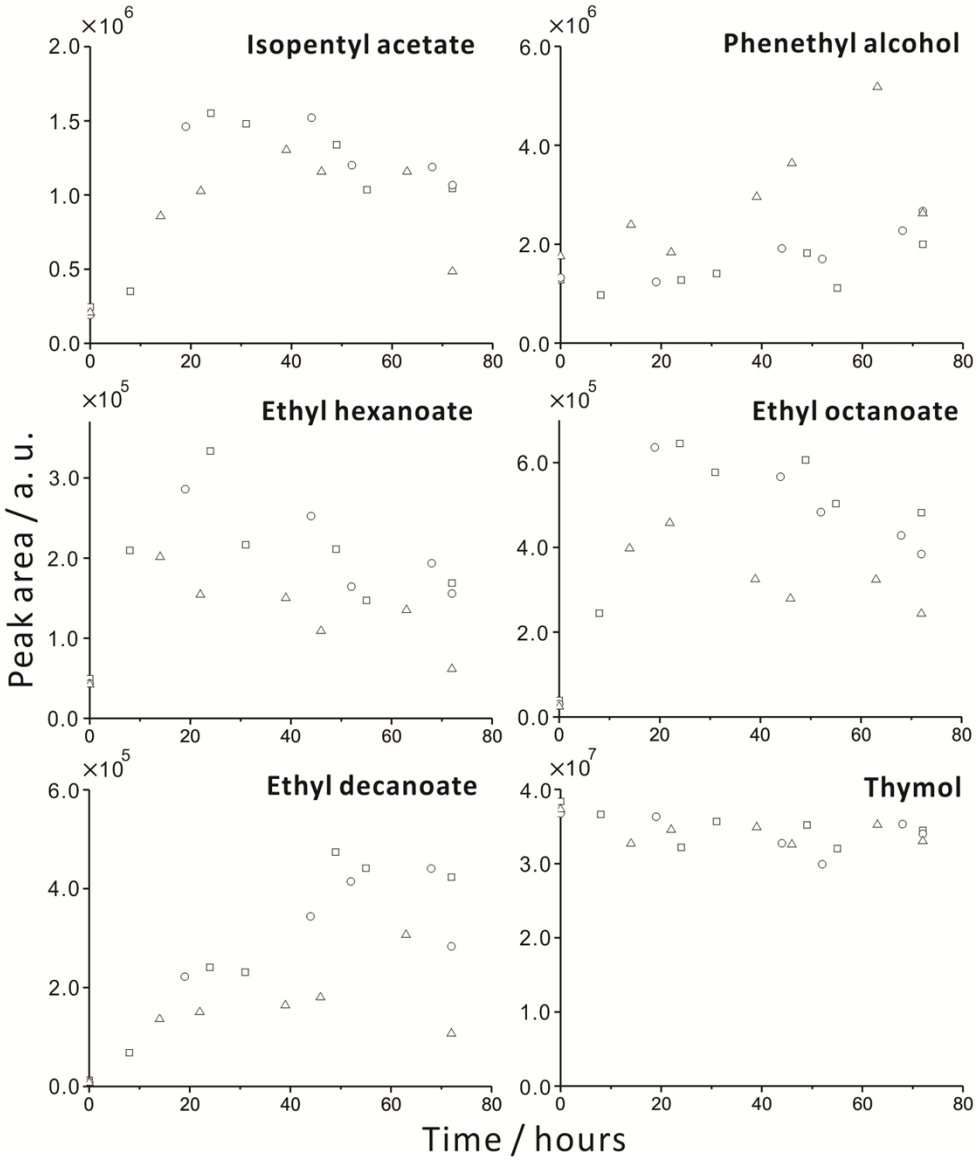

Figure S4. Dynamic monitoring of liquid culture (YM broth) of *S. cerevisiae* by HS-SPME/GC-MS. Incubation temperature: 28 °C. The three symbols (square, circle, and triangle) indicate three experimental replicates.

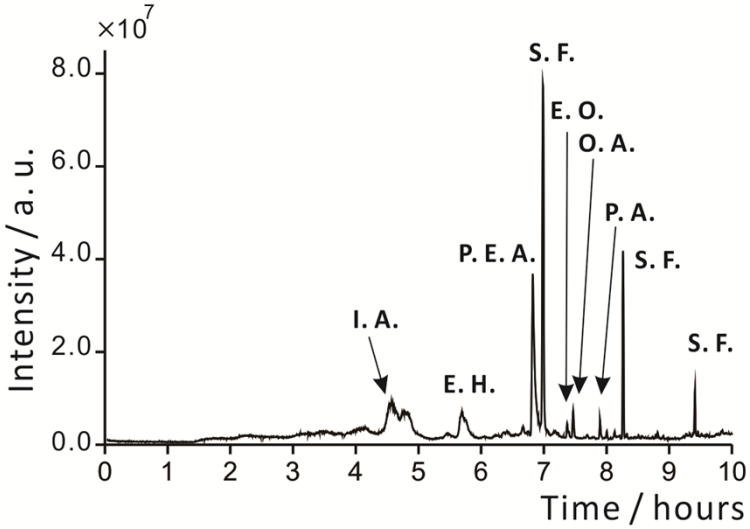

Figure S5. Result of HS-SPME/GC-MS analysis of *S. cerevisiae* culture on agar medium. PTIC (80-130 u e^{-1}) after 24-h incubation at 28 °C. Peaks: I. A., isopentyl acetate; E. H., ethyl hexanoate; P. E. A., phenethyl alcohol; S. F., contaminant related to the SPME fibre; E. O., ethyl oxtanoate; O. A., octyl acetate; P. A., phenethyl acetate.

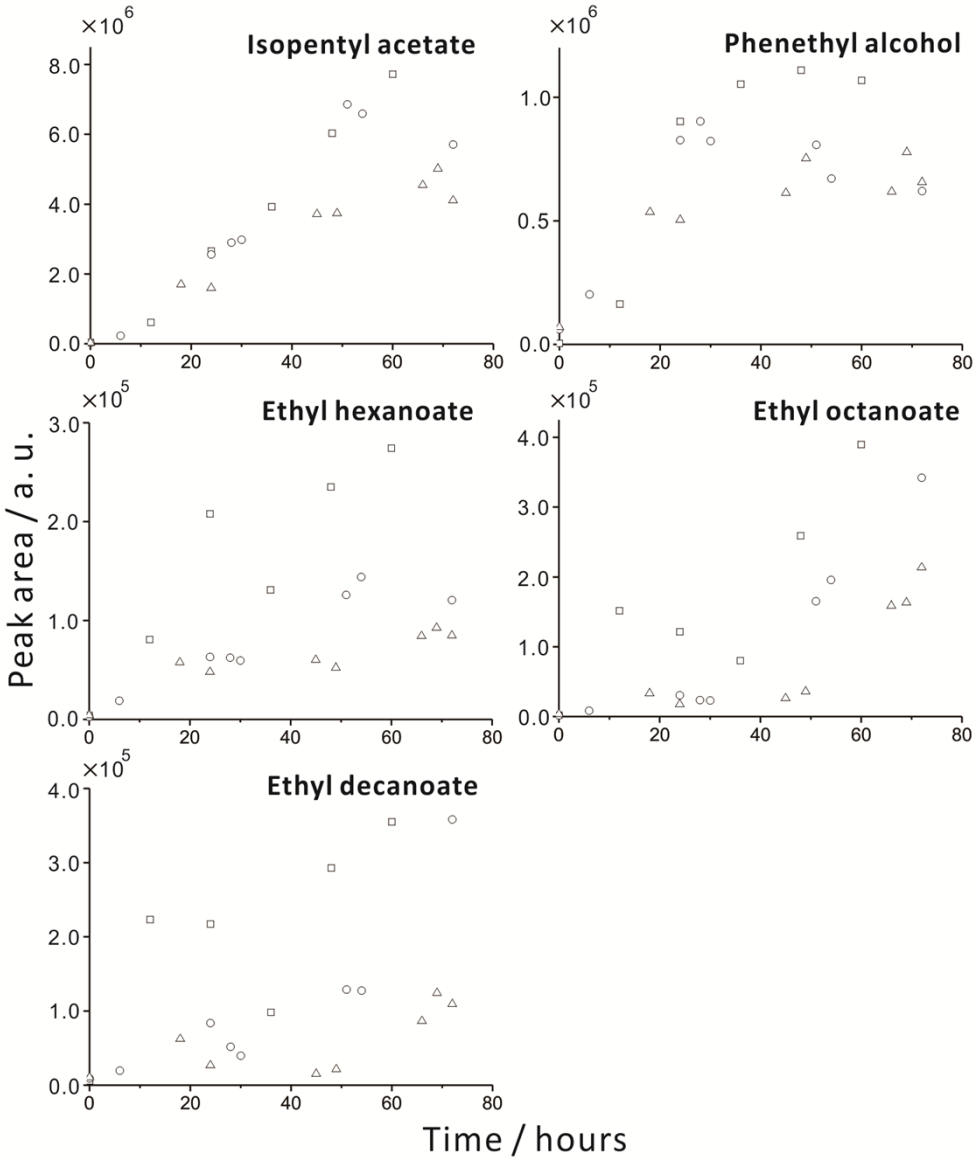

Figure S6. Dynamic monitoring of *S. cerevisiae* culture on agar medium by HS-SPME/GC-MS. Incubation temperature: 28 °C. The three symbols (square, circle, and triangle) indicate three experimental replicates.

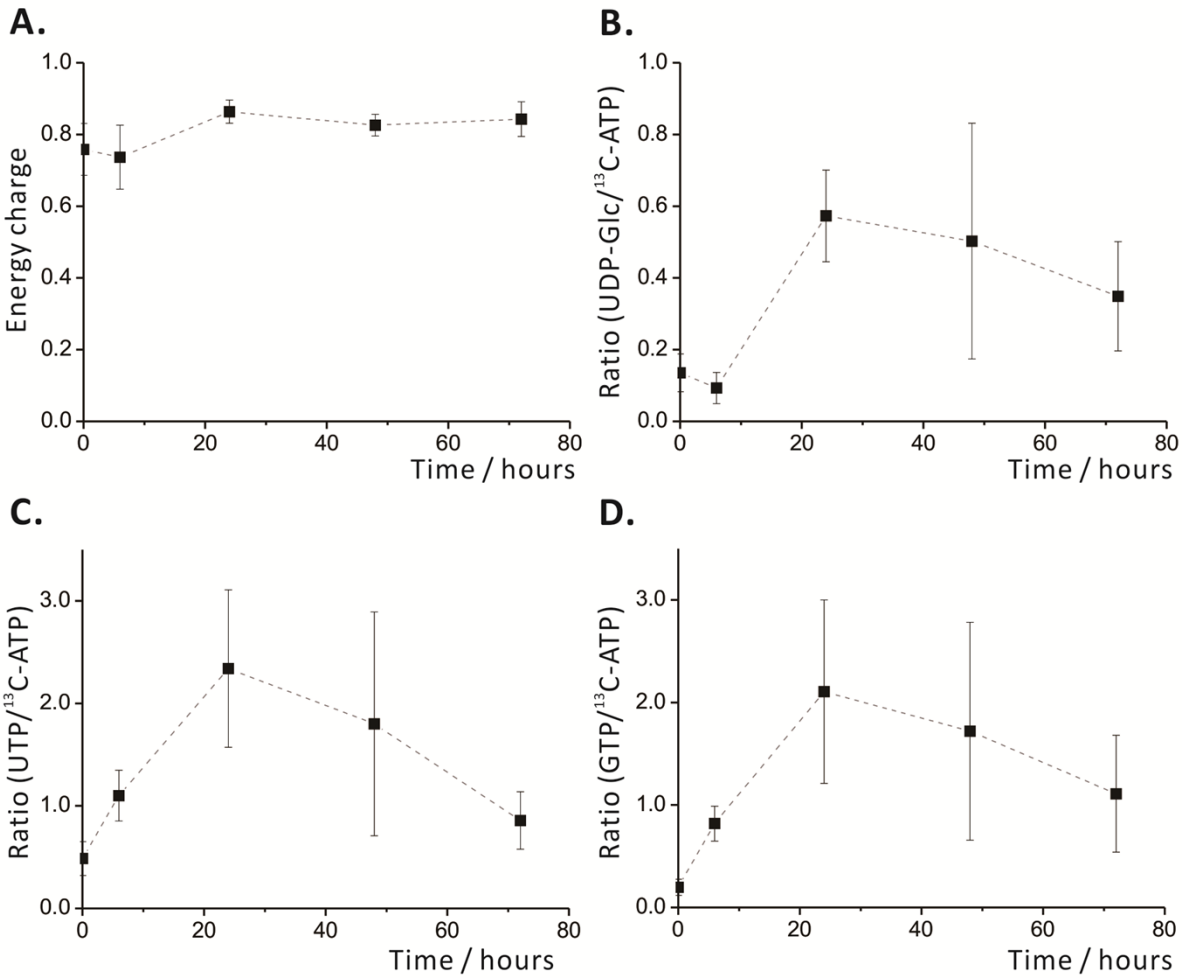

Figure S7. Changes in apparent energy charge and relative abundance of selected intracellular metabolites measured by MALDI-MS: (A) apparent energy charge; (B) UDP-Glc/$^{13}C_{10}$-ATP; (C) UTP/$^{13}C_{10}$-ATP; and (D) GTP/$^{13}C_{10}$-ATP. Incubation temperature: 28 °C. Note well: $^{13}C_{10}$-ATP was used as internal standard. MALDI matrix: 9 mg mL^{-1} 9-aminoacridine dissolved in acetone. Error bars represent standard deviations of 10 technical replicates.

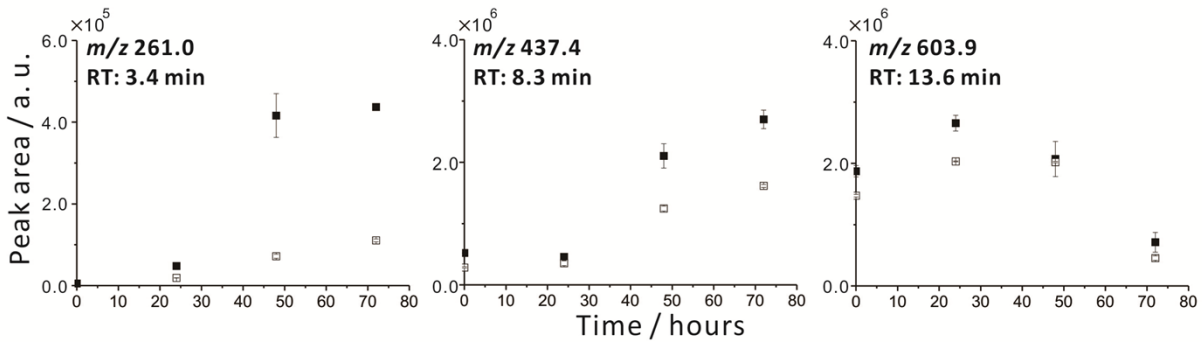

Figure S8. Changes in relative quantities of three selected species in kefir, monitored by reversed phase liquid chromatography coupled to ion trap mass spectrometer with electrospray ion source in the negative-ion mode. Extracted ion currents (EICs) were exported for every *m/z* value (261.0, 437.4, and 603.9 u e^{-1}) with the width of 1.0 u e^{-1}. The EICs were further treated in the PeakFit software to determine peak areas (as described in section 2.7). Solid squares indicate the peak areas obtained from LC-ESI-MS analyses while open squares indicate the peak areas of abundant fragment ions, obtained from LC-ESI-MS/MS analyses (*m/z* 261.0 → 114.0 u e^{-1}; *m/z* 437.4 → 227.1 u e^{-1}; *m/z* 603.9 → 586.3 u e^{-1}). Incubation temperature: 28 °C. All data points are average values from two technical replicates. Error bars correspond to spread.