Construction of synthetic microbiota for reproducible flavor metabolism in Chinese light aroma type liquor produced by solid-state fermentation

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ABSTRACT

Natural microbiota plays an essential role in flavor compounds producing for traditional food fermentation. Whereas, the fluctuation of natural microbiota results in the inconstancy of food quality. Thus, it is critical to reveal the core microbiota for flavor compounds producing and construct a synthetic core microbiota for constant food fermentation. Here, we revealed the core microbiota based on their flavor-producing and co-occurrence performance, using Chinese light aroma type liquor as a model system. Five genera were identified to be the core microbiota, including *Lactobacillus*, *Saccharomyces*, *Pichia*, *Geotrichum*, and *Candida*. The synthetic core microbiota of these five genera presented a reproducible dynamic profile with that in the natural microbiota. Monte Carlo test showed that the interpretation of five environmental factors (lactic acid, ethanol and acetic acid contents, moisture and pH) on the synthetic microbiota distribution were highly significant ($P < 0.01$), which was similar with that in the natural fermentation system. In addition, 77.27% of the flavor compounds produced by the synthetic core microbiota showed a similar dynamic profile ($\rho > 0$) with that in the natural liquor fermentation process, and the flavor profile presented a similar composition. It indicated that the synthetic core microbiota is efficient for reproducible flavor metabolism. This work established a method for identifying core microbiota and constructing a synthetic microbiota for reproducible flavor compounds. It is of great significance for the tractable and constant production of various fermented foods.

IMPORTANCE

The transformation from natural fermentation to synthetic fermentation is essential to construct a constant food fermentation process, which is the premise for stably making high-quality food. According to the functions of flavor-producing and co-occurring in

dominant microbes, we provided a system-level approach to identify the core microbiota in Chinese light aroma type liquor fermentation. In addition, we successfully constructed a synthetic core microbiota to simulate the microbial community succession and flavor compounds production in the *in vitro* system. The constructed synthetic core microbiota could not only facilitate a mechanistic understanding of the structure and function of the microbiota, but also be beneficial for constructing a tractable and reproducible food fermentation process.

KEYWORDS: Chinese liquor, Co-occurring network, Core microbiota, Environmental factors, Flavor compounds, Food fermentation

INTRODUCTION

Traditional fermented foods are usually produced by natural fermentation containing multi-species community (1-4). At present, the transformation from natural fermentation to tractable fermentation with the synthetic core microbiota is essential for consistent quality of fermented foods because only limited genera of microbes in natural microbiota can drive the fermentation process. They not only generate flavor compounds but also maintain microbes' interaction, which serve to achieve the successful food fermentation (5, 6). Thus, revealing the composition of these microbes, that is the core microbiota, is essential for constructing a synthetic microbiota in food fermentation (7).

A series of studies were carried out to identify the core microbiota during food fermentation (7-10). Dominant genera were considered to be an essential component in the food fermentation (11, 12). For example, a total of 17 genera were identified to be dominant microbes due to their relative abundance in cheese (11). However, dominant genera may not have the ability to produce flavor compounds in food fermentation (13, 14). Researchers suggested that the identification of core microbiota should also consider the microbial flavor compounds productivity (7, 15). For example, seven genera were determined as functional core microbiota for production of flavor compounds in Chinese vinegar fermentation (7).

Recently, we found that only the dominant microbes or flavor-producing microbes did not show efficient flavor compounds productivity when they were in a mixed culture (13). Whereas, some other microbes were not flavor compound producers, but they showed the activity to coordinate with those flavor-producing microbes, hence leading to an improvement of flavor compounds (13). For example, *Pichia membranaefaciens* and *Bacillus amyloliquefaciens* were not efficient flavor compound producers, but they alleviated the

competition among flavor compound producers (*Saccharomyces cerevisiae*, *Issatchenkia orientalis* and *Bacillus licheniformis*), and finally altered the producers' growth and flavor compound productions (13). Moreover, the interaction between microbes plays a vital role in some flavor metabolisms, such as 3-(methylthio)-1-propanol and dimethyl disulfide (16). As a consequence, we suggest that besides the flavor compounds productivity, the microbial interaction should also be considered to identify the core microbiota. Moreover, microbial interaction is a critical factor for maintaining the co-occurring in microbial communities, and co-occurring network analysis is an effective tool for studying the microbial interaction (17, 18).

Thus, to overcome the problem of inaccurate definition of core microbiota in fermented foods, we provided a comprehensive method to identify the core microbiota in natural food fermentation, with the combination of flavor-production and co-occurring network analysis. We also took a prudent way to examine the activity of the core microbiota, including its interaction with the environmental factors, and the flavor compound producing. Due to the Chinese light aroma type liquor is a favorite alcoholic beverage and generated by a natural fermentation process (19). In this work, using Chinese light aroma type liquor fermentation as a model system, we provided a strategy to identify the core microbiota and constructed a synthetic microbiota using the core microbiota. Because, Chinese light aroma type liquor, a typical and popular fermented food, is made from spontaneous fermentation involving multiple microbes and complex interactions between microbes (12, 20), and this type of fermentation can produce unique food flavor and taste characteristics (1). And it is also one of the three typical type liquors in China (Sauce aroma, Strong aroma, and light aroma type liquor). In addition, it has a smaller brewing container, shorter fermentation time and easy to observe. So, taking the Chinese liquor as a model system and establishing a method to define the core microbiota are beneficial for constructing a synthetic microbiota to

reveal the mechanism of fermented foods.

RESULTS

Microbial diversity during the fermentation process. Across all samples, altogether 453,217 and 677,563 high-quality sequences were identified for bacteria and fungi after quality control. Meanwhile, a total of 722 and 1,504 operational taxonomic units (OTUs) were obtained for bacteria and fungi with 97% similarity. A total of 49 bacterial genera and 34 fungal genera were identified in the fermentation process (Dataset S1). All the Good's coverage of samples were over 99.80% (Table S1) that indicated sequences represented the majority of microbiota in the fermentation process (21). The average bacterial α-diversity (Chao1 richness and Shannon diversity) declined along with fermentation time on the whole, but there was a fluctuation on day 15 (Table S1). On the contrary, the average fungi α-diversity (Chao1 richness and Shannon diversity) increased along with fermentation time on the whole, but there was a fluctuation on day 5 (Table S1).

As for bacteria (Fig. 1A), at the early stage of fermentation (0 day), Pseudomonas and Bacillus were the predominant genera (average abundance ≥ 10%) (22), whereas *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Weissella*, *Stenotrophomonas*, *Staphylococcus*, *Streptomyces*, *Kroppenstedtia*, *Herbaspirillum*, *Achromobacter*, *Flavobacterium* and *Brevibacterium* were subdominant genera (1% ≤ average abundance ≤ 10%). During middle stage of fermentation (day 5-15), *Lactobacillus* and *Pediococcus* became the predominant genera, and *Leuconostoc* was the subdominant genus at day 15. At the late stage of fermentation (day 20-28), only *Lactobacillus* was the predominant genus, *Pediococcus* became the subdominant genus. As for fungi (Fig. 1B), *Pichia* was the predominant genus in the whole fermentation process. *Geotrichum* (day 5-10) and *Saccharomyces* (day 10) were the predominant genera, and *Saccharomycopsis*, *Rhizopus*, *Clavispora*, *Candida*, *Aspergillus*,

130 *Thermomyces*, *Thermoascus*, *Trichosporon*, and *Lichtheimia* were the subdominant genera at
131 different stages of fermentation.

132 Through statistical analysis of all communities sampled, only 17 bacterial and 16 fungal
133 genera were found at greater than 1% average abundance, which were defined as dominant
134 microbiota (9). A ubiquitously distributed microbiota is usually defined as being present in
135 most samples (9, 23). Therefore, we defined microbes, which exist in more than 50% samples
136 within a total of 14 samples, as ubiquitously distributed microbiota (9). Two genera of
137 bacteria (*Lactobacillus* and *Pediococcus*) and eight genera of fungi *(Pichia*, *Geotrichum*,
138 *Saccharomyces*, *Saccharomycopsis*, *Rhizopus*, *Aspergillus*, *Candida* and *Thermoascus*) were
139 identified to be ubiquitously distributed dominant microbiota (Table S2).
140
141 **Identification of the core microbiota.** Flavor compounds are very important indicators of
142 the liquor quality (24, 25). A total of 41 kinds of flavor compounds were identified during the
143 fermentation (Fig. 2), including four alcohols, two carbonyl compounds, five acids, 20 esters,
144 nine aromatic compounds and one heterocyclic compound.
145 Concentrations of flavor compounds were transformed and converted into a heat map, and
146 hierarchical cluster analysis was achieved. As shown in Fig. 2, the hierarchical clustering
147 results showed that the fermentation process consisted of three-part based on the dynamic
148 profile of flavor compounds: part 1 (day 0), part 2 (days 5-20) and part 3 (days 20-28).
149 Most flavor compounds are related to microbes in food fermentation. Network correlation
150 analysis is a powerful tool to investigate the potential interactions between microbes and
151 flavor compounds (26). Thus, we calculated the Spearman correlation coefficient between 33
152 dominant genera and 41 flavor compounds, and chose the coefficient (ρ) > 0.5 and

significance (P) < 0.05 (27, 28) as strongly correlated nodes of the network (Fig. 3 A). Eight bacterial and seven fungal genera were significantly correlated ($P < 0.05$, $\rho > 0.5$) with 34 kinds of flavor compounds, indicating these 15 genera are the flavor-producing microbiota (Table S3). Among them, *Lactobacillus*, *Saccharomyces*, *Clavispora* and *Candida* were significantly correlated ($P < 0.05$, $\rho > 0.5$) with 26, 26, 16 and 14 kinds of flavor compounds, respectively (Fig. 3 A).

Co-occurrence network analysis allows identifying the co-occurring microbiota (17). We calculated the Spearman correlation coefficient of 33 dominant genera. The Spearman's correlation coefficient ($|\rho|$) > 0.5 and significance (P) < 0.05 was considered to be a valid co-occurrence event (17, 18, 26, 29, 30). Through the co-occurrence network analysis, a total of 25 nodes and 149 edges were obtained ($|\rho| > 0.5$, $P < 0.05$), and the average network clustering coefficient was 0.696, which suggested that the network had modular structures. In Fig. 3 B, different genera are divided into different modular structures. A total of 23 genera presented highly connection (≥ 4 edges per node) (26), and were defined as the co-occurring microbiota, including *Flavobacterium, Lactobacillus, Brevibacterium, Herbaspirillum, Pichia, Staphylococcus, Bacillus, Weissella, Kroppenstedtia, Leuconostoc, Saccharomyces, Aspergillus, Clavispora, Geotrichum, Lichtheimia, Thermoascus, Rhizopus, Achromobacter, Pseudomonas, Stenotrophomonas, Candida, Saccharomycopsis* and *Streptomyces* (Table S4). In the co-occurrence network, *Lactobacillus* and *Saccharomyces* were mainly negatively correlated ($\rho < -0.5$) with other microbes (excepting *Clavispora*), but they showed a positive correlation with each other.

In summary, we obtained ubiquitously distributed dominant microbiota (10 genera), flavor-producing microbiota (15 genera) and co-occurring microbiota (23 genera). Five genera existed in all these three different microbiotas, including *Lactobacillus, Saccharomyces, Geotrichum, Candida* and *Pichia* (Fig. 3 C). Due to their high relative

abundance and frequency, the contributions to flavor productions and the stable microbial network, they were defined as core microbiota in liquor fermentation.

The impact of five environmental factors on the core microbiota were analyzed, including lactic acid content, ethanol content, acetic acid content, moisture and pH (Table S5). Variation partitioning analysis (31) was used to calculate the contributions of these environmental factors. The results showed that these five environmental factors accounted for 87.18% of core microbiota's variation in the *in situ* systems (Table S6). Partial redundancy analysis (RDA) was used to identify the effect of these factors on the core microbiota (Fig. 3 D). Acetic acid content, ethanol content, and lactic acid content were positively correlated with *Lactobacillus*, *Saccharomyces* and *Candida* at the end of fermentation. Monte Carlo replacement test (Table S7) verified the result that these factors were significantly correlated with the core microbiota ($P < 0.05$). It indicated these five environmental factors had a significant influence on the core microbiota.

Reproducible dynamic profile of microbiota in synthetic core microbiota. In this study, we provided a system-level approach to identify the core microbiota in Chinese light aroma type liquor fermentation, and obtained five different core genera during the whole fermentation stage, including *Lactobacillus*, *Pichia*, *Geotrichum*, *Candida* and *Saccharomyces*. Due to the diversity of genera, it was considered to be feasible that isolated species represented certain taxa. For example, cheese rind isolates that represented the most abundant taxa were applied to construct in vitro communities of cheese rind (9). Using 16S and ITS amplification sequence data, when the sequence identity was greater than 99% compared to the type and reference strains, the assignment to the species level was performed (32). Thus, we identified one species with the highest relative abundance in each corresponding genera (Fig. S1 and Table S8) and used as the starter species of the synthetic

203 microbiota, including *Lactobacillus acetotolerans*, *Pichia kudriavzevii*, *Geotrichum*
204 *candidum*, *Candida vini* and *Saccharomyces cerevisiae*. *Lactobacillus acetotolerans* is a
205 functional microorganism in the fermentation of kinds of liquors (Strong aroma, light aroma
206 type liquor and Japanese sake) (32-34). For example, *Lactobacillus acetotolerans* appeared to
207 play a key role during the Chinese strong aroma type liquor fermentation (32), and it had
208 positive relationships with most chemical components that contribute to the quality and flavor
209 of liquor (35). *Pichia kudriavzevii* contribute to the functionality (acids and esters) of foods
210 during fermentation, and it can improve the sensory and some functional properties of the
211 cereal-based substrate during fermentation (36). *Geotrichum candidum*, can produce lipases,
212 which would be important for the productions of fruity aroma compounds (37). *Candida vini*
213 had been shown to contribute to fatty acids (38). Saccharomyces cerevisiae is an important
214 strain of ethanol fermentation in Chinese liquor fermentation (39). Therefore, we chose the
215 five species for the synthetic experiment.
216 We inoculated approximately equal numbers of each species in the five core genera
217 together into fermented grains in the *in vitro* system (Fig. 4 A). *Lactobacillus* became the
218 predominant genus in the *in vitro* system as fermentation proceeded (Fig. 4 A and Fig. S2 B),
219 which was similar with that of the *in situ* system (Fig. S2 A, Fig. S3). *Saccharomyces* and
220 *Pichia* were the dominant genera in the early (1-5 d) and end (28 d) of the fermentation
221 process, which was similar with that of the *in situ* system (Fig. 4 A, Fig. S2 C and Fig. S2 D).
222 *Candida* was the dominant genera in the middle fermentation process (10-25 d). It revealed
223 that the successive direction of the *in vitro* systems (Fig. 4 B) in the principal component is
224 consistent with that of the *in situ* system over a 28 d fermentation period (Fig. 4 C), which
225 demonstrated a highly reproducible microbial succession pattern of the *in vitro* liquor
226 fermentation.
227 The impact of the environmental factors on the synthetic microbiota were also analyzed

(Table S9). Explanations of variation partitioning analysis of the five environmental factors (lactic acid content, ethanol content, acetic acid content, moisture and pH) reached 53.65% in the *in vitro* system (Table S6). This percentage showed that these five factors drove the variation of the synthetic core microbiota. RDA analysis showed that pH was negatively correlated with the other environmental factors that was the same with that in the *in situ* system (Fig. 5 A). Lactic acid content, acetic acid content, ethanol content and moisture were positively correlated with each other that are consistent with that of the *in situ* system. Monte Carlo test also showed that the interpretation of these five environmental factors on the synthetic microbiota distribution were highly significant ($P < 0.01$) (Table S7). Through the change of environmental factors' correlation analysis of the two systems on the temporal dynamics (Fig. 5 B), we found that five environmental factors had a positive correlation ($\rho > 0$) with the core microbiota, especially, moisture, acetic acid content, lactic acid content and pH had a strong correlation ($\rho > 0.6$) between in the *in situ* and *in vitro* systems. These results indicated that the effect of environmental factors on the core microbiota was also similar in the *in vitro* and *in situ* systems.

Reproducible flavor metabolism in synthetic core microbiota. The flavor compound producing in the synthetic microbiota was determined, and 22 flavor compounds were identified in the *in situ* system (Fig. 6 A). The *in vitro* generation of flavor compounds can be divided into three parts (Fig. S4): part 1 (day 0-3), part 2 (day 4-10), part 3 (day 15-28). The temporal dynamics was similar to that in the *in situ* system.

The Spearman correlation coefficient (ρ) of the 22 flavor compounds generation in the two systems was calculated in the fermentation. The result showed that 17 kinds of flavor compounds (ratio = 77.27%) had a positive correlation ($\rho > 0$) with the generation on the temporal dynamics in the two systems (Fig. 6 A). The ratio of different flavor classifications

had similar proportions in both systems (Fig. 6 B), in which the ratio of alcohols and acids accounted for more than 99.85% in the total flavor compounds. These indicated that the flavor metabolism could be reproduced in the *in vitro* system with the synthetic core microbiota.

DISCUSSION

Core microbiota inhabiting in food fermentation is of great importance to the quality and characteristics of foods. Many molecular and ecological approaches have been used to characterize the core microbiota (22, 40-42). In this work, we chose microbial communities in Chinese light aroma type liquor fermentation as a model system and provided a system-level method to identify the core microbiota in natural food fermentation. Taking a prudent way examined the characteristics of all the dynamic succession of the microbiota, the effect of the environmental factors, and the profile of flavor compounds production. Among these compounds, we did not detect the detrimental flavors in Chinese light aroma type liquor fermentation. Most of these flavors have pleasant aromatic smells, such as ethyl acetate (pineapple), ethyl lactate (fruity), 1-Octen-3-ol (mushroom), octanoic acid (cheesy), ethyl 3-phenylpropanoate (floral), γ-nonanolactone (coconut), etc. (43, 44). Although some of these flavors also contain some unpleasant flavors, but these flavors form a special style of products at low concentrations, such as acetic acid (acidic, vinegar), hexanoic acid (sweaty), ethyl oleate (fatty), 3-methyl-1-butanol (malty), etc. (43, 44). We constructed a reproducible synthetic core microbiota, with that of the natural microbiota for liquor fermentation. It would help us to establish a tractable food fermentation system.

In the *in vitro* system, alcohols (without ethanol) and acid contents were a bit higher than those in the *in situ* system (Dataset S2, Dataset S3). Whereas, ester contents were lower than that in the *in situ* system ($P < 0.001$). That may be due to the low concentration of

esterification strains in the *in vitro* system. We also observed slight differences in the microbiota between the *in situ* and *in vitro* systems. For example, a succession of *Saccharomyces* appeared to proceed much more quickly (Fig. S2 C, S2 D), and *Candida* showed a higher relative abundance in the later fermentation in the *in vitro* system (Fig. 4, Fig. S2 D). The difference might result from a higher initial ratio of these genera in the *in vitro* system. Therefore, the initial compositions of the core microbiota should be optimized in the further synthetic core microbiota's fermentation. Different species and different strains of microorganisms under the same genus may have different metabolic functions. Therefore, more functional strains should be isolated. But the same strain in single fermentation and mixed fermentation may show completely different metabolic patterns (16). Therefore, the target functional strains should be synthetically optimized by extensive statistical analysis.

Besides the liquor fermentation system, the methods for identifying the core microbiota and constructing a synthetic microbiota for food fermentation can also be used in a variety of food fermentation processes. Various food fermentations share the members of the core microbiota because these members present similar functions in different food fermentations. For example, *Lactobacillus* was confirmed to be the core microbe in fermentations of vinegar, liqueur, cheese, pickle, and so on (45-47). It contributed amino acids (glutamic acid, alanine, valine, etc.), organic acids (acetic acid, lactic acid, *etc*.), and other flavor compounds (7, 48-50). It would also interact with other microbes, such as *Bacillus*, *Aspergillus* and *Luteococcus*, hence regulate their flavor compounds producing (47, 50-52). *Pichia* was widely used in food fermentation, such as wine and beer fermentation (53-55). It was considered to be an essential producer of esters (56). *Pichia* can also maintain the co-occurring of the community (13), which was similar to that in Fig 3 B. *Geotrichum* can produce lipases, which would be important for the productions of fruity aroma compounds, such as ethyl esters of acetic acid, propionic acid, butyric acid and isobutyric acid (57, 58).

303 *Saccharomyces*, as an ethanol producer, was widely used in liquor and other alcoholic
304 beverages' production (59). It drove the development direction of the microbiota, together
305 with *Lactobacillus* (acid producer) (60, 61). *Candida* was widely used in food fermentations,
306 due to its production of various lipases (antarctica lipase A, rugosa lipases, glucose ester
307 synthesis lipase, *etc*.) (62-64). When *Candida* and *Saccharomyces* were co-cultured in wine
308 fermentation, they produced higher amounts of esters and glycerol, compared with that of
309 single *Saccharomyces* (65). These studies indicated that most of the microbes in the core
310 microbiota had similar functions in different food fermentations.
311 The transformation from natural fermentation to synthetic fermentation is essential to
312 construct a tractable food fermentation process, which is the premise for stably making
313 high-quality foods. We provided a system-level approach to identify the core microbiota in
314 food fermentation and constructed a synthetic microbiota for reproducible flavor metabolism.
315 It would provide a chance for us to define the mechanisms underlying the microbial
316 interaction and contribution to flavor compounds in the food microbiota. It is also important
317 to manipulate the synthetic microbiota and then control the quality of fermented foods.
318
319 **MATERIALS AND METHODS**
320 **Sample collection.** Samples were collected from a local liquor distillery (Shanxi
321 Xinghuacun Fenjiu Distillery Co. Ltd. Shanxi, China,). For liquor fermentation, the steamed
322 grains were mixed with starter at a ratio of 9:1 (w/w) and put into earthenware jars. Then, the
323 jars were sealed for 28 days' fermentation. For the survey of microbial diversity, a total of 12
324 samples (100 g each sample) were collected from 2 jars in the center of the layer (0.5 m deep)
325 at different fermentation times (day 0, 5, 10, 15, 20, and 28) in April 2016. All samples were
326 stored at - 20°C for further DNA extraction and physicochemical parameters determination.
327

328 **DNA extraction, qualification and sequencing analysis.** Each sample (5.00 g) was used
329 to extract genomic DNA using the E.Z.N.A. ® soil DNA Kit (Omega Bio-tek, Norcross, GA)
330 according to manufacturer's instruction. The V3-V4 region of the 16S rRNA bacterial gene
331 was amplified with the universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3) and
332 806R (5'-GACTACHVGGGTWTCTAAT-3') (66). For fungi, the ITS2 region was amplified
333 with the primers of ITS2 (5'- GCTGCGTTCTTCATCGATGC -3') and ITS3 (5'-
334 GCATCGATGAAGAACGCAGC -3') (67). These primers added a set of 8-nucleotide
335 barcodes sequence unique to each sample. The PCR reactions were performed in 25 μL
336 volume, containing 2.5 μL of 10 × Pyrobest Buffer, 2 μL of 2.5 mM dNTPs, 1 μL of each
337 primer (10 μM), 0.4 U of Pyrobest DNA Polymerase (TaKaRa, Takara Holdings Inc.,
338 Nojihigashi, Kusatsu, Shiga, Japan), 15 ng of template DNA, and double-distilled water
339 (ddH_2O) up to 25 μL. Amplification was performed with the previously described method (42,
340 68). Then applicants were pooled into equimolar quantities and subjected to high-throughput
341 sequencing using Miseq Benchtop Sequencer for 2 × 300 bp pair-end sequencing (Illumina,
342 San Diego, CA). Databases of EzBioCloud and Central Bureau of Fungal Cultures
343 (CBS-KNAW) were used for sequence alignment of bacteria and fungus. The fungi and
344 bacterial raw sequences data were deposited in the DNA Data Bank of Japan (DDBJ)
345 database under the accession number of DRA005471 and DRA005916.
346
347 **Population determination by real-time quantitative PCR (qPCR)**. The population of
348 yeast and LAB in liquor fermentation were determined by qPCR. Genomic DNA of samples
349 were used as the templates. For yeast, the sequences were amplified using YEASTF (5'
350 GAGTCGAGTTGTTTGGGAATGC 3') and YEASTR (5'
351 TCTCTTTTCCAAAGTTCTTTTCATCTTT 3') (69) as primers. For LAB, the sequences
352 were amplified using Lac1 (5; AGCAGTAGGGAATCTTCCA 3') and Lac2 (5'

353 ATTYCACCGCTACACATG 3') (70) as primers. qPCR was performed by the StepOnePlus
354 instrument (Applied Biosystems, CA, USA) (16).

355

356 **Sequence processing.** All the raw Miseq-generated sequences were processed via QIIME (V.
357 1.8) (71). Briefly, high-quality sequences was carried out by removing sequences with
358 ambiguous bases > 2, homopolymers > 10, primer mismatches, average quality scores < 20
359 and lengths (excluding the primer or barcode region) < 50 bp. Chimeras were removed using
360 USEARCH (v. 10) (72). The trimmed sequences were clustered into operational taxonomic
361 units (OTUs) with 97% sequence (73), and then calculated Shannon index and Chao1
362 estimator using UCLUST (V. 1.2.22) (74, 75).
363

364 **Analysis of environmental factors and flavor compounds.** Moisture was measured by
365 determining its weight loss after drying 10 g of each sample at 105°C for 3 h (sufficient to
366 ensure constant weight) (76). The pH was measured at a 1:2.5 (w/v) ratio in
367 distillation-distillation H_2O (ddH_2O) with the Laboratory pH meter-FE20 (Mettler Toledo,
368 Shanghai, China) (76). Five-gram samples were added to 10 mL ddH_2O and put in an
369 ultrasonic cleaner (AS30600B, Autoscience, Tianjin, China) for 30 min, and then centrifuged
370 at 8000 × *g* for 10 min. After filtered using a 0.2 μm filter, the filtrate was used to analyze the
371 concentrations of flavor compounds and acids. The flavor compounds content was detected
372 using gas chromatography-mass spectrometry (Agilent 6890N GC system and Agilent 5975
373 mass selective detector, Agilent, Santa Clara, CA) (42). The ethanol content was determined
374 by high-performance liquid chromatography (HPLC, Agilent 1200, Agilent, Santa Clara, CA)
375 using a column aminex HPX-87H (Bio-Rad, Hercules, CA) (77). The contents of lactic acid

and acetic acid were measured using reversed-phase ultra-performance liquid chromatography (UPLC, Waters H-class system, Waters, Milford, MA) with chromatographic column waters Atlantis T3 (4.6 mm×150 mm, 3 μm) (Waters, Milford, MA) and guard column Phenomenex RP-C_{18} Security Guard (4.0 mm×3.0 mm) (Phenomenex Inc. Torrance, CA). The UV detection wavelength was 210 nm. The column temperature was 30°C. The injection volume was 10 μL. The mobile phase was 10 mmol/L NaH_2PO_4 (pH 2.7), and the flow velocity was 0.8 mL/min.

Strains. Predominant microbes were all isolated from the liquor fermentation process, *Lactobacillus acetotolerans*, *Pichia kudriavzevii* and *Candida vini* were deposited in China General Microbiological Culture Collection Center with the accession number of CGMCC No. 14086 and 12418 and 2.2018. *Saccharomyces cerevisiae* was deposited in China Center for Type Culture Collection with the accession number of CCTCC M2014463. *Geotrichum candidum* is a laboratory strain with the number of XY7.

Liquid fermentation. The sorghum extract was used as seed fermentation broth (40). The extract was diluted with distilled water to give a sugar concentration of about 90 g/L and then autoclaved at 115°C for 15 min. 100 mL of medium was added in 150 mL conical flasks, inoculated with a ring of target strain, and then incubated for 48 h at 30°C (yeast) and 24 h at 37°C (LAB). The microscopy was used to continuously count until obtained 10^8 CFU/mL seed fermentation broth. **Solid-state fermentation.** Sorghum (400 g) was added to 500 mL of water in the 3000 L beaker, and mixed the liquefied enzyme (10 U/g) in boiling water (100°C) for 2 h, and then added glucoamylase (50 u/g) maintaining 4 h at 60°C. Reducing sugar of the sorghum extracts about 50 ~ 90 g/kg. The beaker autoclaved at 115°C for 15 min. After cooling, seed fermentation broth was added in the beaker with the cellular population of

401 1×10^5 CFU/g wet sorghum, and then experiments were carried out in 150 mL conical flasks
402 which contained 100 g of sorghum. The flasks were then sealed and incubated at 30°C. In
403 order not to interrupt the fermentation process, 30 flasks were used to fermented according to
404 the above experimental conditions, and three flasks were randomly selected from the same
405 fermentation conditions at 1, 2, 3, 4, 5, 10, 15, 20, 25 and 28 days respectively. After
406 fermentation, the sorghums were used enumeration of different strains, and the rest withdrew
407 and stored at - 20°C for analysis of environmental factors and flavor compounds.

408 **Enumeration of different strains.** After fermentation, 10 g sorghums were added to 25
409 mL phosphate buffer saline (PBS, 0.01M, pH7.2), vortex mix 3000 rpm for 30 s (Dragonlab
410 MX-E, Beijing, China), and under 4 °C for 30 min. The supernatant was gradient diluted and
411 spread plate. Four kinds of yeasts enumeration were carried out on Wallerstein Laboratory
412 nutrient (WLN) medium (78), in which the strains showed different macroscopic features
413 (texture, surface, margin, and color),. *Lactobacillus* enumeration was carried out on MRS
414 Broth (DE MAN, ROGOSA, SHARPE) (34). Standard deviations were calculated from
415 triplicate repetitions of the enumeration.

416 **Statistical analysis.** Standard statistical analyses were conducted with XLSTAT
417 (v.19.02.42992). Heatmap, Variation partitioning analysis, Redundancy analysis (RDA), the
418 Monte Carlo permutation test was calculated by the R program (v. 3.4.0). In the Heatmap,
419 flavor compounds were transformed by z-score. Clustering analysis was performed using the
420 Pearson correlation coefficient, and Euclidean distance based on the flavor compounds
421 content during the fermentation process. The Variation partitioning analysis resulted in five
422 environmental factors and five microbes' average abundance. In constrained ordination,
423 representational difference analysis (RDA) was used to identify the relationship of samples,
424 environmental factors and microbes. The Monte Carlo permutation test was used to examine
425 the significance of the correlation between environmental factors and species distribution. All

the analyses were performed using functions in the Vegan package (v. 2.4-3) (79). The Spearman correlation coefficient (ρ) and Paired-sample t-test were calculated with SPSS Statistics 22, in which $\rho > 0.6$ and $\rho > 0.8$ were representing strongly and highly correlated. The visualization objects of interaction of flavor compounds and microbes and co-occurring analysis were drawn with Gephi (v. 0.9.1) (22).

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FILE S1.

DATASET S1, XLSX file.

DATASET S2, XLSX file.

DATASET S3, XLSX file.

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The authors declare that there are no conflicts of interest.

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666

667 **Figure captions**

668

669 **Fig. 1. Distribution of the relative abundance of genera during the fermentation in the**
670 ***in situ* System.** Only those genera that had an average abundance greater than 1% were
671 indicated. Genera less than 1% abundance were combined and shown in others.

672

673 **Fig. 2. Heatmap of flavor metabolites and hierarchical clustering in the *in situ***
674 **fermentation process.** Flavor compounds were transformed by z-score. Clustering analysis
675 was performed using the Pearson correlation coefficient and Euclidean distance based on the
676 flavor contents during the fermentation process.

677

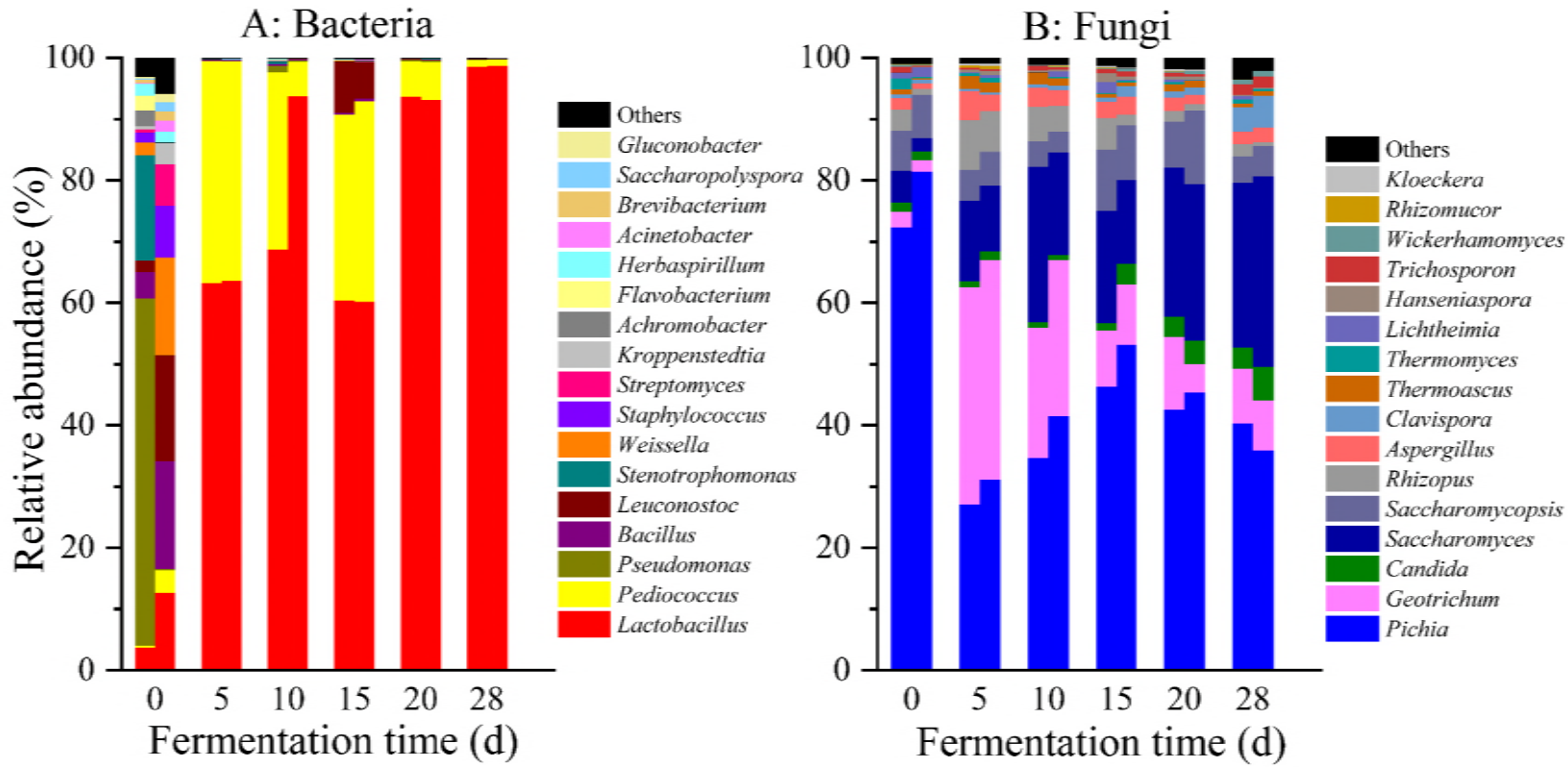
678 **Fig. 3. Identification of the core microbiota in the *in situ* system. (**A) Correlation network
679 between microbial genera and flavor compounds during the fermentation process in the *in*
680 *situ* system. Inner circle nodes represent microbes (light red modules represent bacteria
681 genera, and light blue modules represent the fungi genera), and outer circle nodes represent
682 flavor compounds (different colors represent different flavor types). The thickness of lines
683 are proportional to the value of Spearman's correlation ($\rho > 0.5$, $P < 0.05$). The color of lines
684 are same with the flavor nodes. (B) Correlation network of co-occurring genera in dominant
685 microbiota. Statistically significant ($P < 0.05$) and Spearman correlation coefficient ($|\rho| > 0.5$)
686 indicate the correlations. Light red modules represent bacteria genera, and light blue modules
687 represent fungi genera. Green and red edges indicate negative and positive interaction
688 between genera. The thickness of lines represents the strength of interaction. (C) The Venn
689 diagram of the core microbiota. Different circles represent different genera categories. (D)
690 RDA analysis of fermentation process. Blue dots represent the time of fermentation. Red dots
691 represent the core microbiota. Black arrows represent the different of environmental factors.

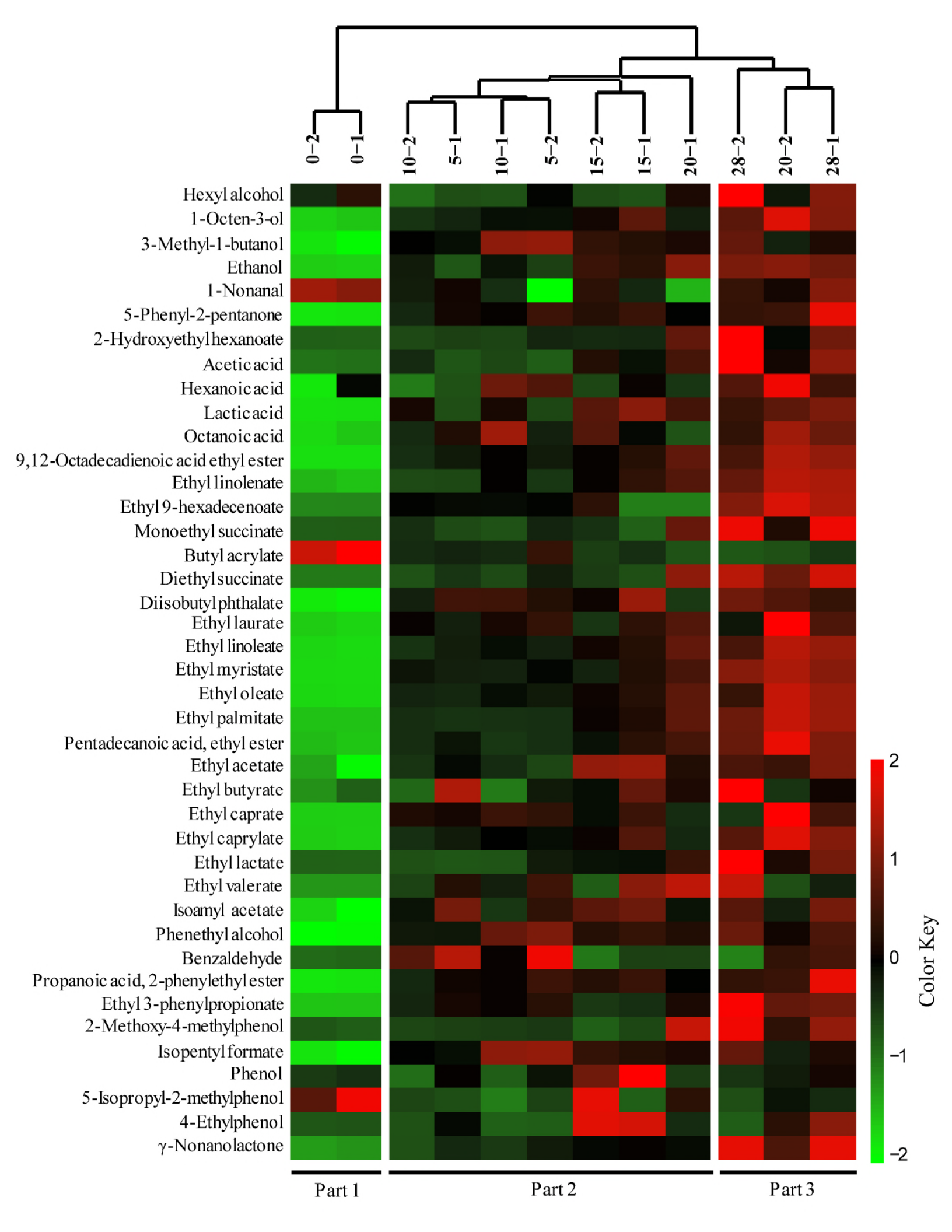
Percentages on the axis represent the eigenvalues of principal components.

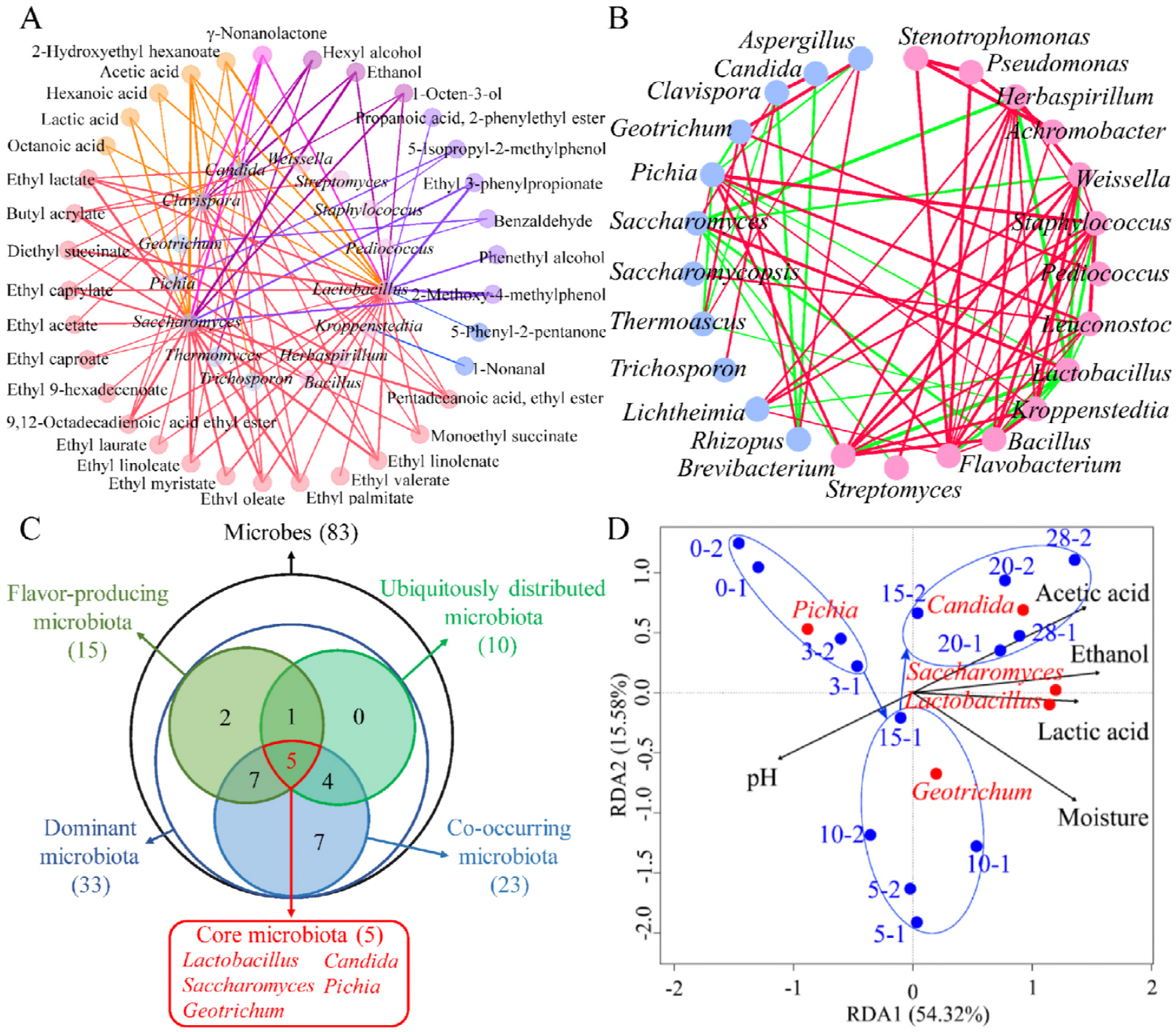
Fig. 4. Reproducible dynamic profile of microbiota in synthetic core microbiota. (A) Distribution of the abundance of genera during the fermentation in the *in vitro* system. (B) *In situ*, the change of principal component in time gradient. (C) *In vitro*, the change of principal component in time gradient.

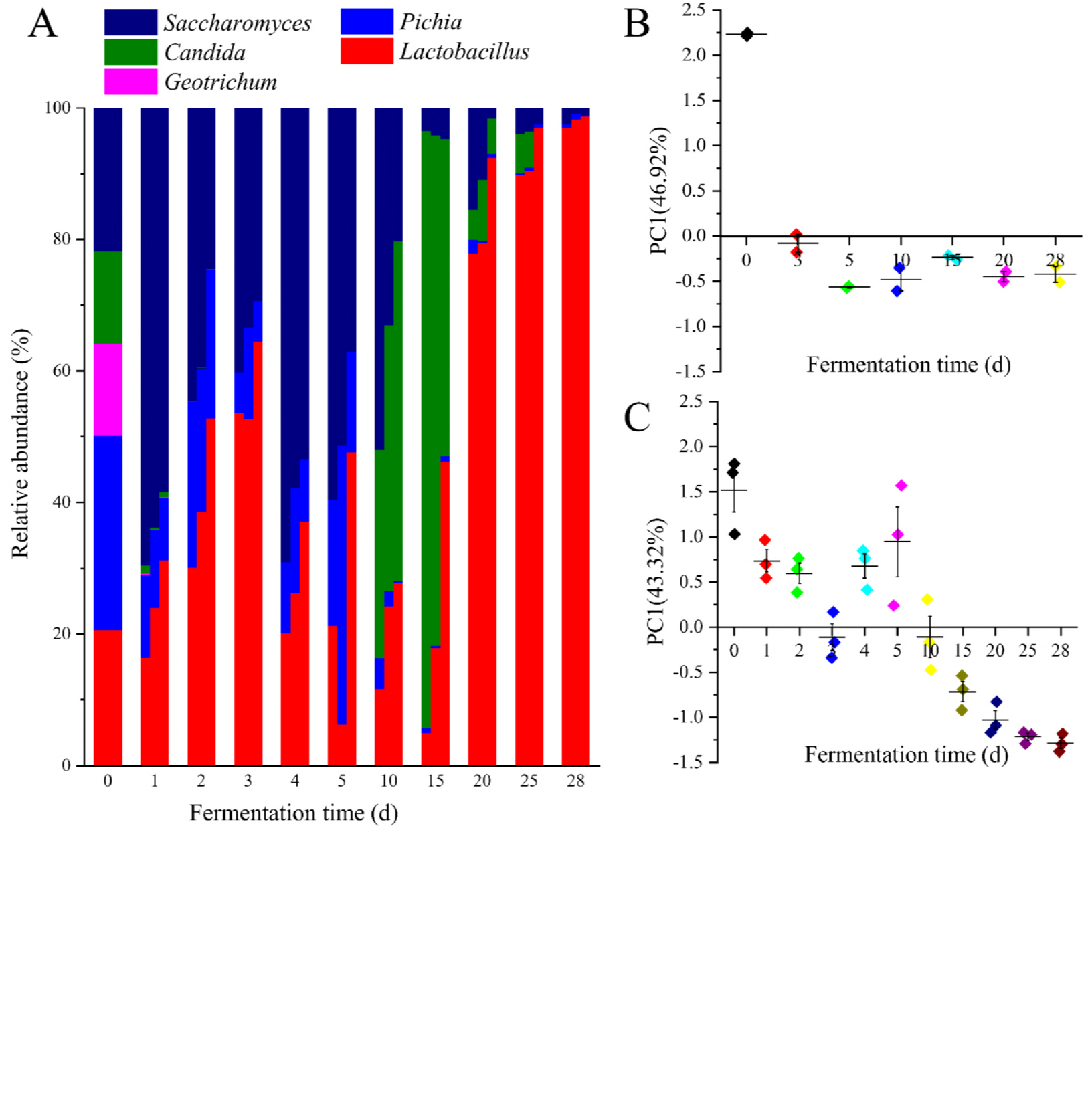
Fig. 5. RDA analysis of fermentation process in the *in vitro* system and the relationship of environmental factors within the *in situ* system. (A) RDA analysis of fermentation process in the *in vitro* system (same with Fig. 3D). (B) The similarity of in the *in situ* and *in vitro* system. The vertical coordinate in the figure represents the Spearman correlation coefficient between the corresponding of environmental factors in the two systems.

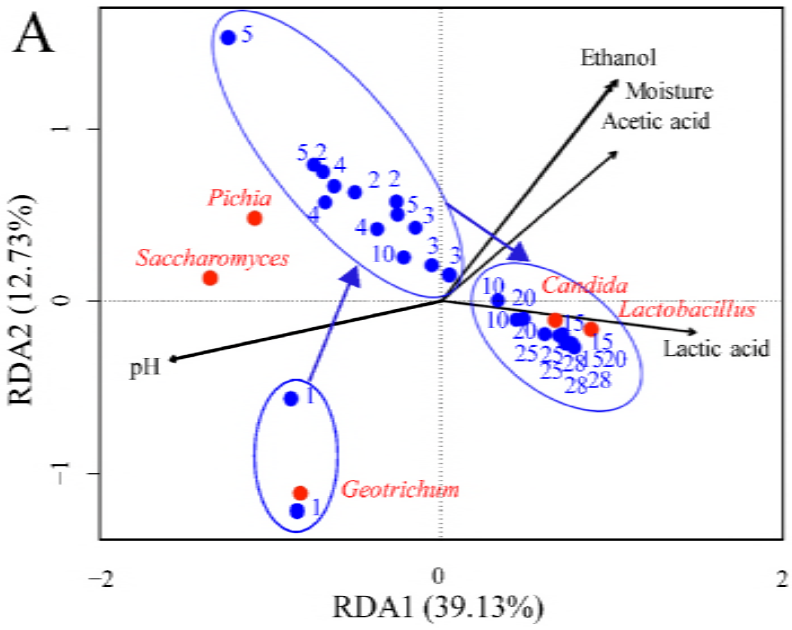
Fig. 6. Reproducible flavor metabolism in synthetic core microbiota. (A) The similarity of two system in 22 kinds of alcohols, acids and esters in two systems. The vertical coordinate in the figure represents the Spearman correlation coefficient (ρ) of the flavor generation along the time axis in the two systems. (B) The ratio of six kinds of flavor compounds in the *in situ* and *in vitro* systems.

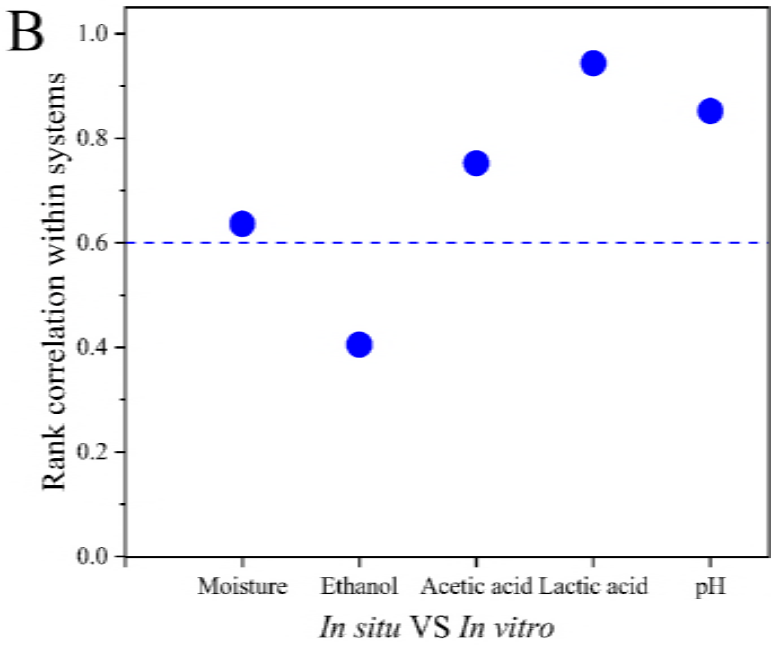

A: Bacteria
B: Fungi
Relative abundance (%)
Fermentation time (d)
0
5
10
15
20
28
20
40
60
80
100
Others
Gluconobacter
Saccharopolyspora
Brevibacterium
Acinetobacter
Herbaspirillum
Flavobacterium
Achromobacter
Kroppenstedtia
Streptomyces
Staphylococcus
Weissella
Stenotrophomonas
Leuconostoc
Bacillus
Pseudomonas
Pediococcus
Lactobacillus
Kloeckera
Rhizomucor
Wickerhamomyces
Trichosporon
Hanseniaspora
Lichtheimia
Thermomyces
Thermoascus
Clavispora
Aspergillus
Rhizopus
Saccharomycopsis
Saccharomyces
Candida
Geotrichum
Pichia

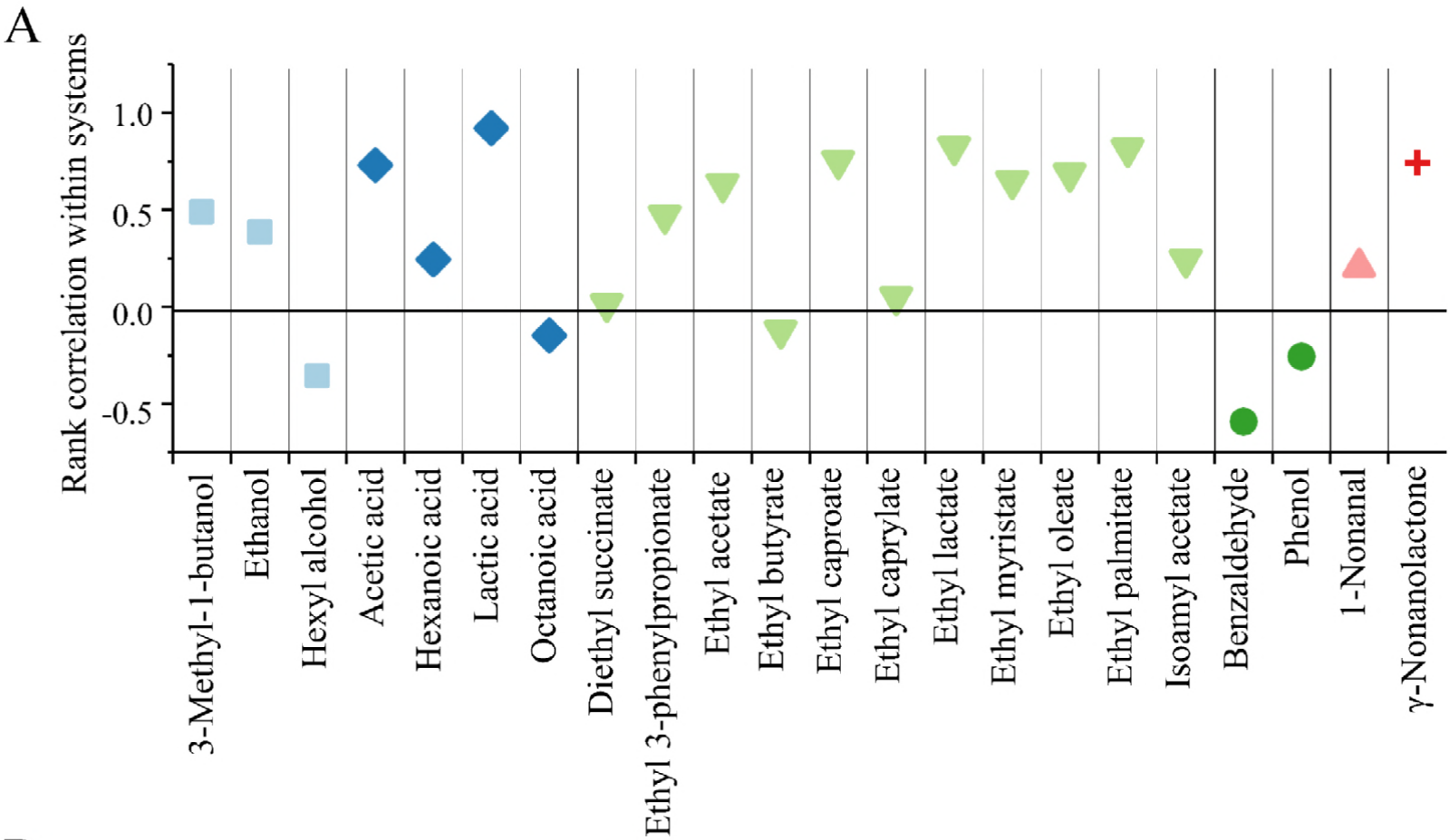

0−2
0−1
10−2
5−1
10−1
5−2
15−2
15−1
20−1
28−2
20−2
28−1
Hexyl alcohol
1-Octen-3-ol
3-Methyl-1-butanol
Ethanol
1-Nonanal
5-Phenyl-2-pentanone
2-Hydroxyethyl hexanoate
Acetic acid
Hexanoic acid
Lactic acid
Octanoic acid
9,12-Octadecadienoic acid ethyl ester
Ethyl linolenate
Ethyl 9-hexadecenoate
Monoethyl succinate
Butyl acrylate
Diethyl succinate
Diisobutyl phthalate
Ethyl laurate
Ethyl linoleate
Ethyl myristate
Ethyl oleate
Ethyl palmitate
Pentadecanoic acid, ethyl ester
Ethyl acetate
Ethyl butyrate
Ethyl caprate
Ethyl caprylate
Ethyl lactate
Ethyl valerate
Isoamyl acetate
Phenethyl alcohol
Benzaldehyde
Propanoic acid, 2-phenylethyl ester
Ethyl 3-phenylpropionate
2-Methoxy-4-methylphenol
Isopentyl formate
Phenol
5-Isopropyl-2-methylphenol
4-Ethylphenol
γ-Nonanolactone
2
1
0
−1
−2
Color Key
Part 1
Part 2
Part 3

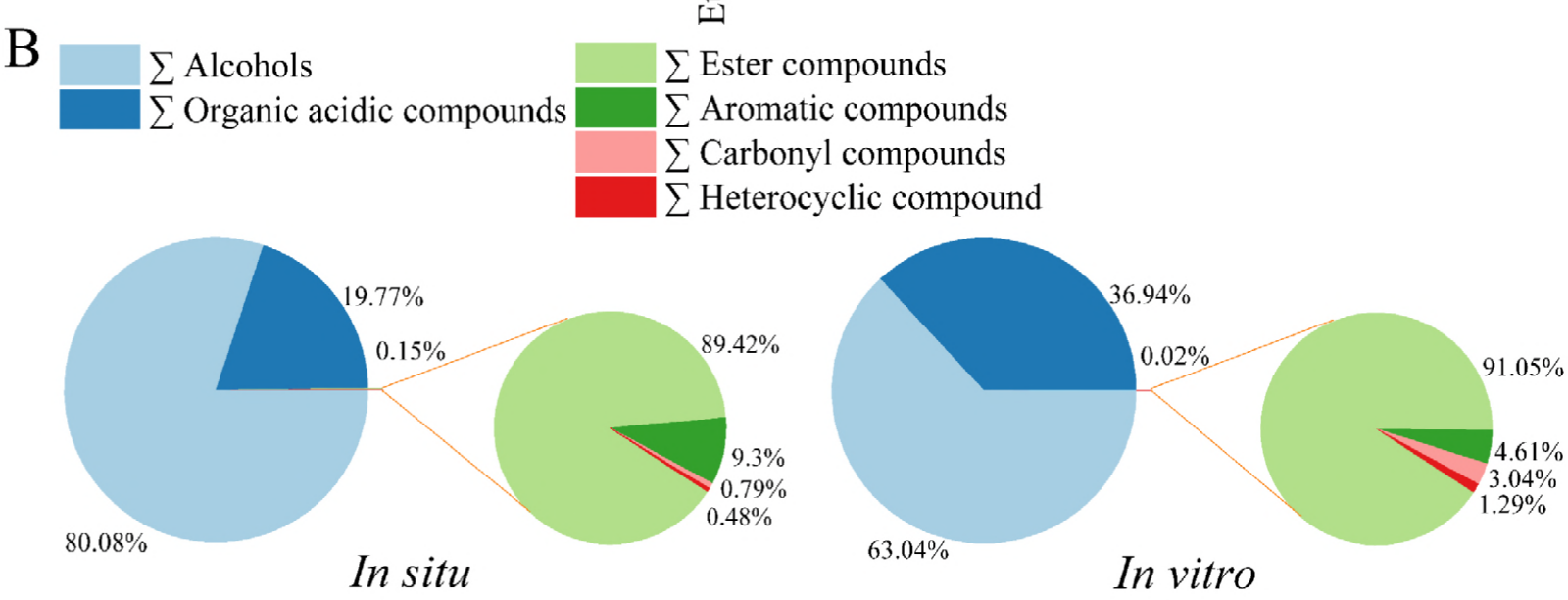

A
γ-Nonanolactone
2-Hydroxyethyl hexanoate
Hexyl alcohol
Acetic acid
Ethanol
Hexanoic acid
1-Octen-3-ol
Lactic acid
Propanoic acid, 2-phenylethyl ester
Octanoic acid
5-Isopropyl-2-methylphenol
Weissella
Candida
Ethyl lactate
Streptomyces
Ethyl 3-phenylpropionate
Clavispora
Butyl acrylate
Staphylococcus
Benzaldehyde
Diethyl succinate
Geotrichum
Pediococcus
Phenethyl alcohol
Ethyl caprylate
Pichia
Lactobacillus
2-Methoxy-4-methylphenol
Ethyl acetate
Saccharomyces
Kroppenstedtia
5-Phenyl-2-pentanone
Ethyl caproate
Thermomyces
Herbaspirillum
1-Nonanal
Ethyl 9-hexadecenoate
Trichosporon
Bacillus
Pentadecanoic acid, ethyl ester
9,12-Octadecadienoic acid ethyl ester
Monoethyl succinate
Ethyl laurate
Ethyl linolenate
Ethyl linoleate
Ethyl valerate
Ethyl myristate
Ethyl oleate
Ethyl palmitate
B
Aspergillus
Stenotrophomonas
Candida
Pseudomonas
Clavispora
Herbaspirillum
Geotrichum
Achromobacter
Pichia
Weissella
Saccharomyces
Staphylococcus
Saccharomycopsis
Pediococcus
Thermoascus
Leuconostoc
Trichosporon
Lactobacillus
Lichtheimia
Kroppenstedtia
Rhizopus
Bacillus
Brevibacterium
Flavobacterium
Streptomyces
C
Microbes (83)
Flavor-producing microbiota (15)
Ubiquitously distributed microbiota (10)
Dominant microbiota (33)
Co-occurring microbiota (23)
2
1
0
5
7
4
7
Core microbiota (5)
Lactobacillus
Candida
Saccharomyces
Pichia
Geotrichum
D
RDA2 (15.58%)
RDA1 (54.32%)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2
-1
0
1
2
0-2
0-1
28-2
20-2
15-2
Acetic acid
Pichia
Candida
20-1
28-1
Ethanol
3-2
3-1
Saccharomyces
Lactobacillus
15-1
Lactic acid
pH
Geotrichum
Moisture
10-2
10-1
5-2
5-1


A
Saccharomyces
Candida
Geotrichum
Pichia
Lactobacillus
Relative abundance (%)
Fermentation time (d)
B
PC1(46.92%)
Fermentation time (d)
C
PC1(43.32%)
Fermentation time (d)


A
Ethanol
Moisture
Acetic acid
Pichia
Saccharomyces
Candida
Lactobacillus
pH
Lactic acid
Geotrichum
RDA2 (12.73%)
RDA1 (39.13%)
−2
0
2
−1
0
1


B
Rank correlation within systems
1.0
0.8
0.6
0.4
0.2
0.0
Moisture
Ethanol
Acetic acid
Lactic acid
pH
In situ VS In vitro


A
Rank correlation within systems
1.0
0.5
0.0
-0.5
3-Methyl-1-butanol
Ethanol
Hexyl alcohol
Acetic acid
Hexanoic acid
Lactic acid
Octanoic acid
Diethyl succinate
Ethyl 3-phenylpropionate
Ethyl acetate
Ethyl butyrate
Ethyl caproate
Ethyl caprylate
Ethyl lactate
Ethyl myristate
Ethyl oleate
Ethyl palmitate
Isoamyl acetate
Benzaldehyde
Phenol
1-Nonanal
γ-Nonanolactone


B
∑ Alcohols
∑ Organic acidic compounds
∑ Ester compounds
∑ Aromatic compounds
∑ Carbonyl compounds
∑ Heterocyclic compound
19.77%
0.15%
80.08%
89.42%
9.3%
0.79%
0.48%
In situ
36.94%
0.02%
63.04%
91.05%
4.61%
3.04%
1.29%
In vitro