Storage time and temperature affect microbial dynamics of yeasts and acetic acid bacteria in a kombucha beverage

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RUNNING HEAD: Kombucha microbiota as affected by storage and temperature

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Highlights

- The microbiota of a kombucha beverage was isolated and molecularly identified
- The community composition of yeasts and AAB was affected by storage and temperature
- *Dekkera anomala* was the dominant yeast species in all samples across storage
- AAB persisted across storage at 4°C and up to 20 days of storage at room temperature

Abstract

Kombucha is a mildly sweet, slightly acidic fermented beverage, commercially available worldwide, that has attracted increasing consumers' interest due to its potential health benefits. Kombucha is commonly prepared using sugared black or green tea, but also other plant substrates are frequently utilised. Kombucha is obtained by fermentation using a symbiotic culture of bacteria

and yeasts, whose composition varies depending on inoculum origin, plant substrates and environmental conditions. After fermentation, kombucha drinks are usually refrigerated at 4 °C, in order to maintain their biological and functional properties. There are no reports on the fate of microbial communities of kombucha in relation to long-term storage time and temperature. Here, for the first time, we monitored the diversity and dynamics of the microbial communities of a kombucha beverage fermented with different herbs during storage at 4 °C and at room temperature, for a period of 90 days, utilising culture-dependent and independent approaches. Moreover, cultivable yeasts and acetic acid bacteria (AAB) were isolated from the beverage, inoculated in pure culture, identified by molecular methods, and yeasts assessed for their functional properties. Total yeast counts were not affected by storage temperature and time, although their community composition changed, as *Saccharomyces* species significantly decreased after 45 days of storage at room temperature, completely disappearing after 90 days. On the other hand, *Dekkera anomala* (*Brettanomyces anomalus*), representing 52% of the yeast isolates, remained viable up to 90 days at both storage temperatures, and was able to produce high levels of organic acids and exopolysaccharides. Data from DGGE (Denaturing Gradient Gel Electrophoresis) band sequencing confirmed that it was the dominant yeast species in all samples across storage. Other yeast isolates were represented by *Saccharomyces* and *Zygosaccharomyces* species. Among AAB, *Gluconobacter oxydans*, *Novacetimonas hansenii* and *Komagataeibacter saccharivorans* represented 46, 36 and 18% of the isolates, whose occurrence remained unchanged across storage at 4 °C and did not vary up to 20 days of storage at room temperature. This work showed that the combination of culture-dependent and independent approaches is important for obtaining a complete picture of the distinctive core microbial community in kombucha beverages during storage, elucidating its diversity and composition, and preliminary characterizing yeast strains with putative functional activities.

Keywords: kombucha microbiota, ITS-RFLP, PCR-DGGE, *Dekkera anomala*, *Zygosaccharomyces*, *Gluconobacter oxydans*, *Novacetimonas hansenii*

1. Introduction

Kombucha is a mildly sweet, slightly acidic fermented beverage reportedly originated in China and Japan, and consumed since 220 BC. It became popular in Russia and Eastern Europe, reached Western Europe and North Africa during World War II, and nowadays is commercially available worldwide (Greenwalt et al., 2000; Zagrabinski, 2020). The increasing interest of consumers in kombucha is ascribed to the occurrence of antioxidant, antimicrobial and hepato-protective compounds, although its health promoting effects lack scientifically sound evidence (Diez-Ozaeta and Astiazaran, 2022; Tran et al., 2020a; Watawana et al., 2015). Kombucha success is demonstrated by its market standing at USD 1.85 billion in 2019 and by the projection of its global market size reaching USD 10.45 billion by 2027 (https://www.fortunebusinessinsights.com/industry-reports/kombucha-market-100230). Currently, the number of kombucha's Brewers International registered companies stand at 150 in the USA and 35 in Europe, representing 69.8% and 16.3% of the total worldwide, respectively (Nyhan et al., 2022).

Kombucha is commonly prepared using sugared black or green tea (*Camellia sinensis*) fermented by a symbiotic culture of bacteria and yeasts (SCOBY) that produce a cellulosic biofilm (pellicle), where they remain embedded. Both SCOBY and the fermented liquid represent the taxonomically diverse matrices utilized as inoculum (Harrison and Curtin, 2021; Tran et al., 2020a). Beyond *C. sinensis* leaves, kombucha beverages are prepared using alternative substrates, such as blackthorn, coffee, goji berries, jasmine, Jerusalem artichoke, lemon balm, milk, mulberry, peppermint, raspberry, sage, thyme, and even molasses from sugar beet processing, with the aim of developing new beverages with novel taste and beneficial properties, deriving also from the

fermenting ingredients (Diez-Ozaeta and Astiazaran, 2022; Emiljanowicz et al., 2020; Jayabalan et al., 2014; Leonarski et al., 2021; Nyhan et al., 2022; Watawana et al., 2015).

Despite the fact that a multitude of symbiotic microbial consortia - whose origins are mostly unknown - are utilized worldwide as starters for the production of kombucha, the most characteristic microorganisms are almost constant across the different studies, consisting of yeasts, acetic acid bacteria (AAB), and occasionally lactic acid bacteria (LAB) (Bishop et al., 2022; Nyhan et al., 2022). Yeasts ferment sugars to ethanol, organic acids and carbon dioxide, then ethanol is further metabolized into organic acids (mainly acetic, gluconic and glucuronic) by the acetic acid microbiota. In addition, yeasts like *Dekkera/Brettanomyces*, contribute to acetic acid production (Tran et al., 2020b). Acetic acid bacteria also use yeast-derived glucose to synthesise bacterial cellulose and gluconic acid (Dufresne and Farnworth, 2000; Greenwalt et al., 1998; Gullo et al., 2018). Recent studies showed that the structure and building of the cellulose biofilm are affected by yeast-AAB interactions (Tran et al., 2021).

During fermentation, the acidity of the beverage increases due to the production of organic acids, responsible for the lowering of pH. In addition, other organic acids are produced, such as lactic, malic, citric and tartaric, known for their antibacterial activity, preventing kombucha spoilage by mesophilic contaminants, together with the low pH (Diez-Ozaeta and Astiazaran, 2022; Neffe-Skocinska et al., 2017; Tran et al., 2020b; Watawana et al., 2015).

The yeast community is mainly represented by *Dekkera/Brettanomyces bruxellensis* (hereafter *D. bruxellensis*), *Dekkera anomala/Brettanomyces anomalus* (hereafter *D. anomala*), *Candida* spp., *Kloeckera* spp., *Pichia* spp., *Saccharomyces cerevisiae*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*, *Torula* spp., *Torulaspora* spp., *Torulopsis* spp., *Zygosaccharomyces bailii, Zygosaccharomyces rouxii,* while bacterial communities are characterized by the acetic acid species *Acetobacter pausterianus*, *Acetobacter aceti*, *Gluconobacter oxydans* and *Komagataeibacter xylinus* (formerly *Gluconacetobacter xylinus*). LAB are sporadically present in kombucha (Bishop et al., 2022; Nyhan et al., 2022).

However, some variations in the composition of microbial communities in kombucha drinks produced worldwide can be ascribed to inoculum origin, plant substrates and to the different geographic, climatic and environmental conditions encountered during fermentation and production processes (Harrison and Curtin, 2021; Jafari et al., 2021; Nyhan et al., 2022; Teoh et al., 2004; Wang et al., 2022).

Interesting shifts were reported in the structure and dynamics of the microbiota during the fermentation period, generally lasting 8-14 days at room temperature (18-28 °C). For example, a culture-dependent study on yeast ecology of kombucha reported that the fermentation was initiated by osmotolerant species, such as *Schizosaccharomyces pombe*, *Torulaspora delbrueckii* and *Zygosaccharomyces bailii*, which decreased with increasing acidity, to be then succeeded by acid-tolerant species (Bishop et al., 2022; Nyhan et al., 2022; Teoh et al., 2004). On the other hand, high-throughput sequencing (HTS) performed on the broth did not detect any variation in the relative abundance of bacterial and yeast genera between days 3 and 10 of the fermentation, being *Gluconacetobacter* and *Zygosaccharomyces* the dominant microbial genera in each of the five samples originated from four different countries (Marsh et al., 2014). Other authors found that the changes in microbial community structure, as revealed by HTS, were paralleled by important changes in the biochemical properties of kombucha beverages (Chakravorty et al., 2016; De Filippis et al., 2018). Recent studies utilising synthetic consortia showed that the metabolic interaction of yeasts and AAB shift kombucha's chemical composition (Tran et al., 2020b).

After fermentation, kombucha drinks are usually refrigerated at 4 °C, in order to maintain their biological and functional properties. Only few works investigated the fate of microbial communities of kombucha drinks in relation to storage time and temperature. Cold storage at 4 °C reduced the viability of acetic acid and lactic acid bacteria, after 14 days: AAB moderately decreased from 9.3×10^6 CFU/mL to 3.4×10^6 CFU/mL while LAB showed a survival rate of 0.98% from the 2nd to 8th day of storage (Fu et al., 2014). Tan et al. (2020) reported that total yeasts and LAB decreased during the 21-day storage of soursop (*Annona muricata*. L.) kombucha at room

129 temperature. Different milk-herbal tea kombucha drinks stored at 4 °C for 30 days showed increases
130 in the number of AAB, and decreases of *Lactobacillus* spp. and *Lactococcus* spp. on the 20th day of
131 storage (Sarkaya et al., 2021). Alas, the only work on the effects of a long-term storage (9 months)
132 on kombucha investigating the variations in some potential beneficial properties, did not analyse the
133 microbiota (La Torre et al., 2021).

134 To the authors' knowledge, there are no reports on microbial composition dynamics of
135 kombucha beverages during long-term storage. Thus, the aim of this study was to monitor and
136 characterize the microbial communities of a kombucha beverage fermented with different herbs
137 during storage at 4 °C and at room temperature, for a period of 90 days. Such storage time was
138 chosen based on our preliminary data on the disappearance of AAB and some yeast genera (data not
139 shown). To this aim, on samples taken at 0, 3, 20, 45 and 90 days we monitored the occurrence of
140 yeasts, acetic acid and lactic acid bacteria during the storage utilising culture-dependent methods
141 and a culture-independent approach, such as PCR-DGGE (Polymerase Chain Reaction-Denaturing
142 Gradient Gel Electrophoresis) analysis of the 16S ribosomal RNA (rRNA) and D1/D2 domain of
143 the 26S rRNA genes, and amplicon sequencing. Moreover, cultivable yeasts and AAB were isolated
144 in pure culture, identified by molecular methods, and yeasts assessed for their functional properties.
145

146 **2. Materials and methods**

147 *2.1. Preparation of kombucha fermented beverage*

148 The kombucha fermented beverage was prepared, by Società Locanda Martinelli di Martinelli
149 Michele & C. S.n.c., Nibbiaia (Livorno), Italy, boiling tap water containing 100 g/L of sucrose and
150 leaving it to cool until 85 °C, then organic green tea and aromatic herbs - *Aloysia citrodora* (lemon
151 verbena), *Malva sylvestris* (mallow), *Rosa* spp. (wild rose), *Mentha* × *piperita* (peppermint) - were
152 added. After 2-hour cooling at room temperature, the herbs were removed by filtration. To promote
153 the fermentation process, the tea was inoculated with 10% of the previously fermented kombucha
154 beverage.

The fermentation was carried out in a dark incubator, covered with a clean cotton cloth, at room temperature (25 °C) for about 4 days, then the beverage was strained through a gauze and poured in dark glass bottles. Finally, bottles were stored at room and refrigerated temperature (4 °C) for 90 days. Samples were collected at the end of fermentation after the final filtration (0 time), and at 3, 20, 45 and 90 days of storage. For each assessment, three bottles were used. pH of the fermented beverage was measured, at each sampling point, using a bench pH-meter (medidor pH basic 20, Crison Instruments, Spain).

2.2. Molecular identification of kombucha microorganisms by culture dependent methods

2.2.1. Microbiological analysis

One millilitre of each sample of the fermented beverage was homogenized with 9 mL of saline-peptone water (9 g/L NaCl, 1 g/L bacteriological peptone, Oxoid, Milan, Italy). Further, a tenfold serial dilution (10^{-1} to 10^{-5}) was carried out in the same solution and aliquots (100 µL) were added in triplicate into a Petri dish containing the agar media listed below. The mesophilic aerobic count was determined on Plate Count Agar (PCA) (Oxoid, Basingstoke, UK) incubated at 30 °C for 72 h (ISO 4833:2003). Yeasts were determined on Wallerstein Laboratory Nutrient (WL) agar (Oxoid, Basingstoke, UK) and Sabouraud Dextrose Agar (SDA) (Oxoid, Basingstoke, UK). To inhibit bacterial grow, both media were supplemented with 100 mg/L chloramphenicol and incubated at 28 °C for 5 days.

AAB were analysed on Yeast Peptone Mannitol agar [YPM, 5 g/L Yeast extract (Oxoid, Basingstoke, UK), 3 g/L peptone (Sigma-Aldrich, St. Louis, MO, USA), 25 g/L mannitol (Sigma-Aldrich, St. Louis, MO, USA), 20 g/L agar (Sigma-Aldrich, St. Louis, MO, USA)] and on WL agar. The media were added with 100 mg/L cycloheximide and 5 mg/L penicillin and incubated at 30 °C for 48 h under aerobic conditions.

LAB were counted on Man Rogosa Sharpe (MRS) agar (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1 g/L of Tween 80 (Sigma-Aldrich, St. Louis, MO, USA) and 5 mg/L of amphotericin B to inhibit yeast growth, and incubated for 96 h at 30 °C under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK).

SPSS version 23 (IBM Corp., Armonk, NY, USA) was used for one-way ANOVA statistical analyses of microbial counts expressed as Log CFU/mL. In particular, for each storage period, microbial count of samples maintained at room temperature and those of samples maintained at 4 °C were compared using the Student *t*-test ($P<0.05$).

2.2.2. Isolation of yeasts and AAB

Samples of the kombucha fermented beverage were used to isolate yeasts and AAB in pure culture. Yeasts grown on WL agar medium, allowing the macroscopic discrimination of colonies based on their color and/or morphology (Pallmann et al., 2001; Tran et al., 2020b), were randomly selected based on phenotypic colony characteristics and growth rate, then purified by streaking four times and further characterized. AAB colonies were randomly selected, then purified by streaking four times and further characterized. Each strain was named with the acronym of the Collection of the Department of Agriculture, Food and Environment of the University of Pisa (IMA, International Microbial Archives), followed by the letter "K" and a progressive number, plus "Y" or "AAB" for yeasts or bacteria, respectively. Purified strains were stored at -80 °C in the appropriate broth medium, supplemented with 20% (w/v) glycerol.

2.2.3. Molecular identification of yeast isolates

A preliminary classification of yeasts was made on the basis of the growth rate, the morphology of the colonies and the observation under the optical microscope. Subsequently, different molecular methods were used for the genotypic identification of yeasts.

Yeast colonies, characterized by a slow rate growth, along with the reference strain *D. anomala* ATCC 10562, were analysed by direct colony PCR with pA1 (5'-TATAGGGAGAAATCCATATAAAAC-3) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers, specific for *Dekkera anomala* (Egli and Henick-Kling, 2001). Reaction was carried out in a final volume of 25 µL. In particular, a small sample of growing cells was picked and transferred in a mix containing 2.5 µL of 10× Ex Taq Buffer (Takara Biotechnology), 0.2 mM of each dNTP (Takara Biotechnology), 1 µM of each primer (Eurofins) and heated to 95 °C for 15 mins. Then 1.25 U of Takara Ex Taq polymerase (Takara Biotechnology) was added to the PCR mix reaction and the amplification was carried out with an iCycler-iQ Multicolor Real-Time PCR Detection System (Bio-Rad) using the following conditions: initial denaturation at 94 °C for 1 min followed by 30 cycles at 94 °C for 1 min, 52 °C for 2 min, 72 °C for 1 min, with final extension at 72 °C for 5 min. Amplification products were analysed by electrophoresis on 1.5% (w/v) agarose gels stained with 0.5 µg/mL REALSAFE Nucleic Acid Staining (Real laboratory SL, Valencia, Spain) in Tris-borate-EDTA (TBE) buffer (Sigma-Aldrich, Milan, Italy) at 80V for 1h. A 100 bp DNA ladder (Thermo Scientific™) was used as a molecular weight marker.

The remaining yeast isolates were analysed by 5.8S-ITS regions amplification. DNA was extracted from microbial liquid cultures grown at 28 °C using "Master Pure™ Yeast DNA Purification Kit" (Epicentre®). Amplification reaction was carried out in a final volume of 50 µL, using ITS1 (5′-TCC GTA GGT GAA CCT GCG G3′) and ITS4 primers (White et al., 1990) and containing 5 µL of 10× r Taq Buffer (Takara Biotechnology), 0.2 mM of each dNTP (Takara Biotechnology), 0.5 µM of each primer (Eurofins), 1.25 U of Takara rTaq polymerase (Takara Biotechnology) and 10–20 ng of DNA. PCR amplifications were carried out with an iCycler-iQ Multicolor Real-Time PCR Detection System (Bio-Rad) using the following conditions: initial denaturation at 94 °C for 1 min followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, with final extension at 72 °C for 5 min. Amplification products were analysed by electrophoresis, as described above.

231 Isolates producing an ITS fragment corresponding to that of the genus *Saccharomyces* (850
232 bp), were analysed by Restriction Fragment Length Polymorphism (RFLP) analysis of the amplified
233 5.8S-ITS regions in order to discriminate species in the *Saccharomyces* sensu stricto complex.
234 Amplicons were digested at 37 °C overnight using *Hae*III and *Hpa*II (BioLabs, Ipswich, MA, USA)
235 enzymes. Fragments were separated on 2% (w/v) agarose gels and electrophoresis was performed as
236 described above.
237 All gels were visualized by UV and captured as TIFF format files by the UVI 1D v. 16.11a
238 program for FIRE READER V4 gel documentation systems (Uvitec Cambridge, Eppendorf).
239 The identification of isolates was confirmed by sequencing the D1/D2 domain of the 26S
240 rRNA gene. The amplification was carried out using NL1 (5'-
241 GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3')
242 primers (Kurtzman and Robnett, 1998), as reported by Palla et al. (2020). Amplicons were than
243 purified using the QIAquick PCR Purification Kit (Qiagen), quantified and 5′ sequenced by
244 Eurofins Genomics (Ebersberg, Germany). Sequences were analysed using BLAST on the NCBI
245 web (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The related sequences were collected and aligned
246 using MUSCLE (Edgar, 2004a, 2004b), and phylogenetic trees were constructed using the
247 Maximum Likelihood method based on the kimura 2-parameter model (Kimura, 1980) in Mega 11
248 (Tamura et al., 2021) software (http://www.megasoftware.net/) with 1000 bootstrap replicates. The
249 sequences were submitted to GenBank (https://submit.ncbi.nlm.nih.gov) (Benson et al., 2013) under
250 the accession numbers from ON783040 to ON783063.
251
252 *2.2.4. Molecular identification of AAB isolates*
253 As the 36 AAB isolates showed only one colony morphology, 11 were randomly selected for
254 further molecular characterization. DNA was extracted from microbial liquid cultures grown at 30
255 °C using "MasterPure™Yeast DNAPurification Kit" (Epicentre®) according to the manufacturer's
256 protocols. AAB isolates were identified by 16S rRNA gene amplification using 27f (5′-GAG AGT

TTG ATC CTG GCT CAG-3′) and 1495r (5′-CTA CGG CTA CCT TGT TAC GA- 3′) primers (Lane, 1991; Weisburg et al., 1991). Amplification reactions were carried out in a final volume of 50 µL, containing 5 µL of 10× Ex Taq Buffer containing Mg2+ (Finnzymes), 0.2 mM of each dNTP (EuroClone), 0.2 µM of each primer (Eurofins Genomics), 0,625 U di Taq DyNAzyme II DNA polymerase (Finnzymes) and 10–20 ng of DNA. PCR amplifications were carried out with an iCycler-iQ Multicolor Real-Time PCR Detection System (Bio-Rad) using the following conditions: 95 °C initial denaturation for 2 min; 35 amplification cycles of 1 min and 20 s at 94 °C, 1 min at 54 °C, 1 m and 30 s at 72 °C; final extension at 72 °C for 5 min. The presence of amplicons was confirmed by electrophoresis in 1.5% (w/v) agarose gel and electrophoresis was performed as described in the section 2.2.3. The identification of isolates was confirmed by sequencing AAB 16S gene amplicons and PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), quantified and 5′ sequenced by Eurofins Genomics (Ebersberg, Germany). The sequences were submitted to GenBank (https://submit.ncbi.nlm.nih.gov) (Benson et al., 2013) under the accession numbers from ON729432 to ON729442.

2.3. Molecular identification of kombucha microorganisms by culture independent methods (PCR-DGGE)

2.3.1. DNA extraction of samples and PCR amplification

Genomic DNA was extracted from 10 mL kombucha samples using DNeasy® PowerSoil Kit® (QIAGEN Group, Germantown, MD) according to the manufacturer's protocol. The extracted DNA was stored at -20 °C until further analyses.

For the analysis of yeast communities, a fragment of D1/D2 region of the 26S rRNA gene was amplified using NL1 (5′-GCC ATA TCA ATA AGC GGA GGA AAA G-3′) and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′) primers (Cocolin et al., 2000), while for the analysis of AAB communities, the V7-V8 region of 16S rRNA gene was amplified with the WBAC1 (5′- GTC GTC AGC TCG TGT CGT GAG A -3′) and WBAC2 (5′-CCC GGG AAC GTA TTC ACC GCG - 3′)

283 primers (Lopez et al., 2003). An additionally GC clamp (5′-GCG GGC CGC GCG ACC GCC GGG
284 ACG CGC GAG CCG GCG GCG G-3′) and (5′-CGC CCG GGG CGC GCC CCG GGC GGC
285 CCG GGG GCA CCG GGG G-3′) was added to the primer NL1 and WBAC2, respectively. PCR
286 amplifications were performed using 10– 20 ng of DNA as described by Palla et al. (2017).
287 Amplification conditions were: 94 °C initial denaturation for 1 min; 35 amplification cycles of 30 s
288 at 94 °C, 30 s at annealing temperature, 30 s at 72 °C; final extension at 72 °C for 5 min. The
289 annealing temperatures for yeasts and bacteria, were 55 and 60 °C, respectively. The presence of
290 amplicons was confirmed by electrophoresis in 1.5% (w/v) agarose gel as described in the section
291 2.2.3.
292
293 *2.3.2. DGGE, profile analyses and band sequencing*
294 For DGGE analyses, 20 µL of amplicons were separated in 8% (w/v) polyacrylamide gels with a
295 36–58% and 36-60% urea-formamide gradient, for yeasts and AAB, respectively, using the
296 DCode™ Universal Mutation Detection System (Bio-Rad, Milan, Italy). Taking into account the
297 results of isolates identification, a composite mix of yeast 26S rRNA gene fragments from *Z. lentus*
298 IMA K36Y, *S. uvarum* IMA K29Y, *Z. bailii* IMA K32Y, *S. cerevisiae* IMA K8Y, *D. anomala* IMA
299 KDY and a composite mix of bacterial 16S rRNA gene fragments from *G. oxidans* IMA KG AAB,
300 *K. saccharivorans* IMA K62 AAB, *N. hansenii* IMA K17 AAB were added on each side and in the
301 center of DGGE gels as reference DGGE markers (M). Gels were run at 90 V and 60 °C for 16 h,
302 stained for 30′ in 500 mL of TAE buffer 1X containing 50 µL of Sybr Gold Nucleic Acid Gel Stain
303 (Thermo Fisher Scientific, Italia) and visualized as previously described.
304 DGGE profiles were digitally processed with BioNumerics software version 7.6 (Applied
305 Maths, St-Martens-Latem, Belgium) and microbial community composition was assessed by cluster
306 analysis of DGGE profiles, as reported in Palla et al. (2018). Similarities between DGGE patterns
307 were calculated by determining Pearson's similarity coefficients for the total number of lane
308 patterns from the DGGE gel using the band matching tool with an optimization of 1%. The

similarity coefficients were then used to generate the dendrogram utilizing the clustering method UPGMA (Unweighted Pair Group Method Using Arithmetic Average).

The main bands of DGGE profiles were excised from the gels for sequencing at the Eurofins Genomics MWG Operon (Ebersberg, Germany) as reported in Palla et al. (2017). DNA was extracted and re-amplified using primers without the GC clamp. PCR products were than purified, quantified and sequenced as previously described. Sequences were analysed using BLAST on the NCBI web (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The related sequences were collected and aligned using MUSCLE (Edgar, 2004a, 2004b), and phylogenetic trees were constructed using the Maximum Likelihood method based on Jukes-Cantor model (Jukes and Cantor, 1969) for yeasts and on Kimura 2-parameter model (Kimura, 1980) for AAB in Mega 11 software (Tamura et al., 2021) (http://www.megasoftware.net/) with 1000 bootstrap replicates. The sequences were submitted to GenBank (https://submit.ncbi.nlm.nih.gov) (Benson et al., 2013) under the accession numbers from ON797641 to ON797659 for yeasts and from ON720146 to ON720159 for AAB.

2.4. Qualitative functional characterization of yeast isolates

All the yeasts isolated from the kombucha fermented beverage were functional characterized using "in vitro" analyses. The analyses were performed in triplicate.

The capacity to produce organic acids was determined by inoculating, on WL agar, 10 µL (10^6 CFU) of yeast liquid cultures grown at 25 °C. Plates were than incubated at 25 °C for 24 hours and visual evaluated for the presence of halo zone around colonies as described by Palla et al. (2021). Protease activity of yeasts was assessed on YEPD without Peptone containing 2% skim milk (Oxoid, Basingstoke, UK) as described by Palla et al. (2017). The capacity to produce exopolysaccharides (EPS) was evaluated by pick-test analysis (Ricciardi et al., 1997; Zotta et al., 2022). Yeasts were grown on YEPD agar added with 20 g/L of sucrose (Carlo Erba, Milan, Italy) and incubated at 25 °C for 24 hours. At the end of incubation the colonies were tested for compactness or ropiness by touching them with a sterile inoculation loop.

335

336 **3. Results**

337 *3.1. Effects of different storage times and temperatures on the microbiota of the kombucha*
338 *fermented beverage, as assessed by culture dependent methods*

339 *3.1.1. Microbiological analysis*

340 In order to study the microbiological changes in the fermented beverage during 90 days of storage
341 at room temperature and at 4 °C, the microbial groups of biotechnological interest were analysed:
342 yeasts, AAB and LAB. Total mesophilic aerobic counts, used as a parameter to determine the
343 microbial quality of the beverage, and the acidity of the drink were also evaluated. In particular, the
344 fermented beverage was analysed at the end of fermentation after straining, and after 3, 20, 45 and
345 90 days of storage. Total mesophilic aerobic counts on PCA medium (Table 1, Supplementary Fig.
346 S1) resulted about 6.8 Log CFU/mL in all samples at 0 and 3 days storage. After 45 and 90 days
347 storage, samples maintained at room temperature showed significant decreases of 3 and 4 Log,
348 respectively, compared to samples maintained at 4 °C, which showed values of 5.26 ± 0.11 Log
349 CFU/mL.

350 The pH of the beverage did not significantly vary during the storage period, both at room
351 temperature and at 4 °C, ranging from 3.28±0.1 and 3.46±0.0.

352 Concerning the microbial groups of biotechnological interest, microbiological analyses
353 showed that both yeast and AAB counts were not affected by the medium used. In particular, total
354 yeast counts were not significantly affected by storage temperature and time, showing a mean value
355 of 6.73 ± 0.09 Log CFU/mL (Table 1).

356 On the basis of colony growth rate, it was possible to discriminate large yeast colonies,
357 growing in about 48 hours (fast-growing yeasts, FGY), from small colonies growing in about 5 days
358 (slow-growing yeasts, SGY). Microbiological analyses highlighted that SGY were predominant
359 during all the storage period, independently by the temperature, showing constant counts of about
360 6.72 ± 0.09 Log CFU/mL (Table 1). The viable count of FGY at the beginning of the storage period

(0 day) was about 5.36 ± 0.13 Log CFU/mL, remaining stable, during the 90 days of storage, for all samples maintained at 4 °C. Samples maintained at room temperature showed a decrease of about 2 Log after 45 days and no colonies were detected at 90 days of storage (Table 1, Supplementary Fig. S1). FGY counts of the latter samples resulted significantly lower than those of the samples maintained at 4 °C for 45 and 90 days (of about 2 and 5 Log, respectively).

The dynamics of AAB was consistent with FGY counts, as their number at the beginning of the storage period (0 day) was about 4.50 Log CFU/mL, remaining stable, during the 90 days of storage, for all the samples maintained at 4 °C. Conversely, the counts of AAB found in the samples maintained at room temperature showed a slight decrease during the first 20 days, followed by a statistically significant decrease after 45 days (1.53 ± 0.23 and 0.53 ± 0.00 Log CFU/mL on WL agar and YPM, respectively). No AAB colonies were detected after 90 days of storage (Table 1, Supplementary Fig. S1).

The number of LAB in the kombucha fermented beverage was lower than that of AAB and yeasts at 0 day storage (2.30 ± 0.14 Log CFU/mL). No LAB were detected after 3 and 20 days of storage in samples maintained at room temperature and 4 °C, respectively (Table 1, Supplementary Fig. S1).

3.1.2. Yeasts and AAB isolation and molecular identification

A total of 58 yeasts were randomly isolated, based on growth rate and phenotypic colony characteristics on WL agar medium, and cellular features under light microscope. Fast-growing colonies, including 19 isolates (IMA K1Y, K8Y, K20Y, K24Y-K26Y, K28Y-K30Y, K35Y, K42Y-K44Y, K46Y, K47Y, K49Y, KCY, KEY, KRY), were characterised by large cream to green umbonate colonies, and ellipsoid cells, ascribed to *Saccharomyces* genera (Fig. 1a), while slow-growing colonies, including 39 isolates (IMA K2Y-K7Y, K10Y-K19Y, K21Y-K23Y, K27Y, K31Y-K34Y, K36Y-K41Y, K45Y, K48Y, KDY, KFY, KHY, KNY, KOY, KPY, KQY) were characterised by small cream, smooth cupular colonies, and ogival cells, possibly ascribed to

387 *Dekkera* spp. (Fig. 1b). As shown by our preliminary experiments (data not shown) and previous data
388 reporting that *Dekkera anomala* was the predominant species in green tea kombucha beverages
389 (Coton et al., 2017), the DNA of the 39 SGY isolates, along with the reference strain *D. anomala*
390 ATCC 10562, was amplified by species-specific PCR using the *D. anomala* primers pA1 and ITS4
391 (Egli and Henick-Kling, 2001). An amplicon of about 450 bp was obtained from 30 isolates (IMA
392 K2Y-K7Y, K10Y-K19Y, K21Y-K22Y, K27Y, K33Y-K34Y, K37Y-K39Y, KDY, KFY, KHY,
393 KNY, KOY, KPY), as for the reference strain *D. anomala* ATCC 10562 (Table 2, Supplementary
394 Fig. S2). On the contrary, the DNA of the other 9 isolates was not amplified. The identification of
395 such isolates, along with the 19 characterized by a fast rate growth and the *D. anomala* IMA KDY,
396 was carried out, after DNA extraction, by amplification of the ITS region with the primers ITS1 e
397 ITS4 (White et al., 1990). Results showed that all the 19 yeast isolates characterized by a fast rate
398 growth produced a 850 bp ITS amplicon while among the slow rate growing isolates, 7 (IMA
399 K31Y, K32Y, K41Y, K23Y, K40Y, K45Y, KQY) produced a 780 bp amplicon and 2 (IMA K36Y,
400 K48Y) a 700 bp amplicon (Table 2, Supplementary Fig. S3). The 19 isolates producing an ITS
401 amplicon of about 850 bp were therefore identified as belonging to the genus *Saccharomyces*
402 (Esteve-Zarzoso et al., 1999) and then subjected to restriction fragment length polymorphism
403 (RFLP) analysis, using *Hae*III and *Hpa*II enzymes, in order to identify the species. Six isolates
404 (IMA K8Y, K26Y, K28Y, K43Y, K46Y, KRY), displayed a restriction pattern of 325, 230, 170 and
405 125 bp with *Hae*III and of 725 and 125 bp with *Hpa*II (Table 2, Supplementary Fig. S4). Such
406 profiles corresponded to those of *S. cerevisiae* (Agnolucci et al., 2007). The remaining 13 isolates
407 (IMA K1Y, K20Y, K24Y, K25Y, K29Y, K30Y, K35Y, K42Y, K44Y, K47Y, K49Y, KCY, KEY),
408 displayed a restriction pattern of 495, 230 and 125 bp with *Hae*III and of 725 and 125 bp with
409 *Hpa*II. Such profiles were reported to correspond to those of *S. bayanus/pastorianus* (Fernàndez-
410 Espinar et al., 2000). Among such isolates, seven showed two weak additional fragments, revealing
411 a polymorphism within the ITS sequences, consistently with their hybrid status (Sampaio et al.,
412 2017) (Table 2, Supplementary Fig. S4).

For the isolates identified as *D. anomala* (30) and *S. cerevisiae* (6) one representative of each group (IMA KDY and K8Y, respectively), along with the remaining 22 isolates, were subjected to amplification of the D1/D2 region of 26S rDNA and subsequent sequencing (Tables 2, 3). The results allowed the identification of all the 58 yeast isolates, corresponding to *D. anomala* (30), *S. cerevisiae* (6), *S. uvarum* (6), *S. uvarum/S. bayanus/S. bayanus/pastorianus* (7), *Zygosaccharomyces bailii* (3), *Zygosaccharomyces parabailii* (4) and *Zygosaccharomyces lentus* (2) (Fig. 2, Tables 2-3). The molecular identification confirmed the presumptive characterization based on phenotypic traits, showing that *D. anomala* and *Zygosaccharomyces* species corresponded to the slow-growing yeasts, and *S. cerevisiae* corresponded to the fast-growing yeasts.

Among 36 AAB isolated from the two media which showed the same translucid colony aspect, 11 were randomly selected for further molecular characterization at species level by 16S region rRNA gene amplification and subsequent sequencing. Despite the uniformity of colony morphology, the results allowed the identification of three AAB species corresponding to *Gluconobacter oxydans* (5), *Novacetimonas hansenii* (former *Komagataeibacter hansenii*) (Brandão et al., 2022) (4) and *Komagataeibacter saccharivorans* (2) (Table 3).

3.2. Effects of different storage times and temperatures on the microbiota of kombucha fermented beverage, as assessed by culture independent methods

The yeasts and AAB community dynamics of the kombucha fermented beverage were monitored during storage at 4 °C and at room temperature for a period of 90 days by PCR-DGGE.

For the analysis of yeast communities, a DNA fragment of approximately 250 bp of the partial D1/D2 domain of 26S rRNA gene was successfully amplified from all kombucha samples. DGGE analyses of PCR products of the different samples showed similar patterns, characterized by a distinctive intensity of fragments (Fig. 3). The yeast community composition and its dynamics during storage at 4 °C and at room temperature for a period of 90 days were studied by cluster analysis of DGGE profiles (Fig. 4). The dendrogram showed two main clusters, with a similarity of

75%. The first cluster included kombucha samples stored at room temperature for 45 and 90 days, with a similarity of 95%, while the other one included all the other samples, with a similarity of 89%. In particular, the latter was formed by two sub-clusters, in which samples stored at 4 °C for 45 and 90 days, with a similarity of 93%, were separated from samples stored at both temperatures, for 0, 3 and 20 days, with a similarity of 93%. Such results highlighted that temperature highly affected yeast community composition of the kombucha fermented beverage after 45 and 90 days of storage.

In order to identify the yeast species, PCR-DGGE bands were excised, sequenced and affiliated to species by using BLAST and phylogenetic trees analyses (Fig. 5). Yeast communities characterizing the kombucha beverage at 0, 3 and 20 days storage were represented by *D. anomala*, *S. cerevisiae*, *S. uvarum/S. eubayanus/S. pastorianus*, *Z. bailii/Z. parabailii/Z. pseudobailii* and *Z. lentus*, both at room and cold temperature. By contrast, after 45 and 90 days storage, temperature modulated the occurrence of *Saccharomyces* species, which strongly decreased when samples were stored at room temperature, consistently with the data obtained by culture-dependent methods (Supplementary Fig. S1). Moreover, the DGGE molecular fingerprinting showed that *D. anomala* was the dominant yeast species in all samples across the storage (Fig. 3).

For the analysis of AAB communities, a DNA fragment of approximately 330 bp of the V7-V8 region of 16S rRNA gene was successfully amplified, with different intensity, for all kombucha samples, with the exception of those stored for 45 and 90 days at room temperature. The AAB community composition and its dynamics during storage at 4 °C and at room temperature for a period of 90 days were analysed by cluster analysis of DGGE profiles (Figs. 6, 7). The dendrogram showed a main cluster, separated with a similarity of 57% from samples stored at room temperature for 45 and 90 days. The main cluster was further split into two sub-clusters, where the samples stored for 45 and 90 days at 4 °C grouped separately (76% of similarity) from those stored for 0, 3 and 20 days. In the latter cluster, samples maintained at room temperature for 20 days clustered separately at 79% of similarity from all the other samples. Overall, the storage temperature of the

kombucha fermented beverage affected AAB community composition starting from 20 days of storage at room temperature (Figs. 6,7).

In order to identify AAB, PCR-DGGE bands were excised, sequenced and affiliated to species by using BLAST and phylogenetic trees analyses (Fig. 8, Table 3). The AAB communities characterizing the kombucha beverage stored at 4 °C were represented by *Gluconobacter* sp., *N. hansenii, K. saccharivorans*, *Komagataeibacter medellinensis/intermedius*, *Komagataeibacter maltaceti/europeus*, *Komagataeibacter oboediens/rhaeticus* across storage.

3.4. Qualitative functional characterization of yeast isolates

In order to select functional yeasts and make them available as starters for the production of health-promoting fermented foods and beverages, the yeast isolated from our kombucha beverage were preliminary assessed "in vitro" for their ability to produce organic acids, exopolysaccharides and to hydrolyze proteins (Rai et al., 2019). The 58 yeast isolates were able to produce organic acids, although only 20% of them at high levels, 63% at medium levels and 17% at low levels (Table 4). Among high producers (halo zone ≥ 7 mm) there were 11 isolates belonging to *D. anomala* species and the isolate *S. uvarum* IMA K20Y.

Pick-test allowed the detection of 29% of isolates able to produce exopolysaccharides. Among them, 76% belonged to *D. anomala*, 12% to *Z. parabailii,* 6% to *Z. bailii* and 6% to *Z. lentus*. The isolates *D. anomala* IMA K27Y, *D. anomala* IMA K37Y and *D. anomala* IMA KPY, showed the highest activity (Table 4).

Regarding protein hydrolysis, high protease activity was shown by 7% of the isolates (Table 4); in particular, the isolates *Z. bailii* IMA K41Y, IMA K32Y, *Z. parabailii* IMA K40Y and *Z. lentus* IMA K32Y, showed the highest activity (halo zone ≥ 6 mm). It is interesting to note that such activity was completely absent in *D. anomala* species.

4. Discussion

490 This is the first study, to the best of our knowledge, monitoring microbial community diversity and
491 dynamics during kombucha long-term storage, using both culture-dependent and independent
492 techniques. Total yeast counts were not affected by storage temperatures and times, although their
493 community composition changed after 45 and 90 days of storage at room temperature. Interestingly,
494 AAB counts did not vary up to 20 days of storage at room temperature. A distinctive core microbial
495 community was unravelled, mainly represented by *D. anomala* which remained viable across
496 storage up to 90 days and was able to produce high levels of organic acids and exopolysaccharides
497 "in vitro".
498
499 *4.1. Effects of different storage times and temperatures on the microbiota of the kombucha*
500 *fermented beverage by culture dependent methods*
501 In this work, total microbial counts did not differ from those reported in the literature at the end of
502 kombucha fermentation, ranging from 6.8 Log CFU/mL to 5.4 CFU/mL after the end of the
503 fermentation process (Chen and Liu, 2000; Tan et al., 2020; Teoh et al., 2004). Total yeasts viable
504 counts were comparable to those reported for kombucha during fermentation and after 14 days of
505 refrigerated storage (Fu et al., 2014; Teoh et al., 2004; Tran et al., 2020b) and maintained their
506 viability across the storage, up to 90 days, when conserved at 4 °C. Accordingly, yeast numbers did
507 not change after 30 days of storage at 4 °C, in milk, milk-green tea and milk-blackberry kombucha
508 fermented and pasteurized beverages, which were supposed to represent a good nutrient rich
509 environment for yeasts (Sarkaya et al., 2021). By contrast, when the kombucha beverage was stored
510 at room temperature, only *D. anomala* survived, probably due to its ability to withstand stressful
511 environments and lack of nutrients (Steensels et al., 2015). Previous works reported the decrease of
512 yeast counts during 21 days storage period at room temperature in soursop kombucha (Tan et al.,
513 2020).
514 AAB populations remained unchanged across the storage period at 4 °C, and up to 20 days
515 at room temperature. This finding is consistent with previous data on the persistence of AAB during

30 days storage at 4 °C in four different milk/herbal kombucha fermented and pasteurized beverages (Sarkaya et al., 2021). Interestingly, AAB were still cultivable, on the different microbiological substrates, even after 45 days at room temperature, although colony number significantly decreased, as compared with 0 time. Then they disappeared after 90 days storage. Unfortunately, no previous data can be found in the literature, for a comparison. However, AAB counts at the end of the fermentation process, about 4 Log CFU/mL, were comparable to those reported by Chen and Liu (2000). The data on the persistence of AAB up to 90 days are intriguing, given their obligate aerobic status, although some works reported a similar behaviour across a shorter time period, 14 and 30 days (Sarkaya et al., 2021; Tran et al., 2020b). The latter Authors suggested that their medium might possess a level of dissolved oxygen sufficient to allow AAB growth. However, we cannot discuss this item, as the dissolved oxygen concentrations were not measured in our samples.

The high counts of AAB even after 90 days in the refrigerated kombucha beverage and after 20 days at room temperature is an interesting finding, as these bacteria, positively modifying the environment for yeasts, contribute to the production of diverse metabolites alleged to provide health benefits, despite the lack of clinical evidence (Diez-Ozaeta and Astiazaran, 2022; Fu et al., 2014; Nyhan et al., 2022). Such data could boost further studies aimed at evaluating the possibility of energy-sustainable storage of kombucha drinks.

The level of LAB (2.3 Log CFU/mL) at the end of the fermentation process was lower than that reported previously, but consistent with their disappearance, after 8 days storage at 4 °C (Fu et al., 2014). Other works showed the erratic occurrence of LAB in kombucha, where they were absent (Gaggìa et al., 2019; Neffe-Skocinska et al., 2017), present in low numbers (Chakravorty et al., 2016; De Filippis et al., 2018; Marsh et al., 2014) or, occasionally, present in high abundance in liquid medium during green tea fermentations (Coton et al., 2017).

The pH of the kombucha beverage, varying from 3.28 to 3.46, was in the range of the values reported in the literature, placing it within acid foods where most spoilage microorganisms are

542 unable to grow (below a pH of 4.0) (Greenwalt et al., 1998, 2000; Hrnjez et al., 2014; Nummer,
543 2013).
544
545 *4.2. Yeasts and AAB isolation and molecular identification*
546 The 58 yeast isolates, analysed by molecular methods, were identified as to *D. anomala* (52%), *S.*
547 *cerevisiae* (10%), *S. uvarum* (10%), *S. uvarum/S. bayanus/S. bayanus/pastorianus* (12%), Z. *bailii*
548 (5%), Z. *parabailii* (7%) and *Z. lentus* (4%). Such yeast species did not differ from those isolated
549 from diverse kombucha drinks worldwide: for example, *D. anomala* was the predominant species in
550 our kombucha beverage, consistently with previous findings (Coton et al., 2017; Reva et al., 2015).
551 The high occurrence of *D. anomala* confirms its ability to adapt both to the acidic environment, as it
552 was the predominant yeast in milk kefir granules from different Italian regions (Garofalo et al.,
553 2015), and to the limited availability of nutrients, as the genus *Dekkera*/*Brettanomyces* has been
554 reported to prevail in nutrient-poor environments (Laureys et al., 2020). Indeed, different species
555 have been isolated from beer, whose pH is similar to that of kombucha. It is interesting to note that
556 the presence of *D. anomala* and *D. bruxellensis* in fermented drinks such as wine, cider and beer
557 was considered negatively, as these two yeast species are able to produce volatile phenols affecting
558 drink flavours (Agnolucci et al., 2017; Buron et al., 2011, 2012; Smith and Divol, 2016). However,
559 in other fermented beverages, like lambic beer, *D. anomala and D. bruxellensis* participate in the
560 spontaneous fermentation process and are supposed to positively affect the development of the
561 unique sensorial characteristics, by producing acetic acid, thus increasing the flavour complexity of
562 the beer (Steensels et al., 2015). During kombucha fermentation, these two *Dekkera* species,
563 together with AAB, contribute to acetic acid production, limiting the level of ethanol, whose high
564 concentrations could affect microbial growth (Coton et al., 2017; Tran et al., 2020b).
565 The genus *Zygosaccharomyces,* well represented in our beverage, was previously described
566 as the dominant genus in kombucha sourced from Canada, UK, USA, Ireland, Australia and Turkey
567 (Arikan et al., 2020; Marsh et al., 2014; Teoh et al., 2004). In particular, the species *Z. bailii* is

568 known to be well adapted to the environment, being capable of tolerating high sugar and acetic acid
569 concentrations (Coton et al., 2017; Thomas and Davenport, 1985). Interestingly, *Z. bailii* was one of
570 the non-*Saccharomyces* yeasts isolated from kombucha and investigated for their suitability in
571 alcohol-free beer production (Bellut et al., 2018). Here, in addition to *Z. bailii*, we isolated also the
572 phylogenetically related species (hybrid) *Z. parabailii* (Suh et al., 2013), that was identified during
573 icewine fermentation by culture-dependent methods and in experimental and commercial
574 kombuchas (Andreson et al., 2022; Gaggía et al., 2019; Li et al., 2018). Although in wine
575 fermentation *Z. bailii* was in some cases considered as a spoilage agent, it was proposed as starter,
576 together with *S. cerevisiae*, in order to enhance wine taste, body and aroma (Domizio et al., 2011;
577 Garavaglia et al., 2015).

578 The genus *Saccharomyces* was sub-dominant in our kombucha beverage and strongly
579 decreased after 45 days of storage, completely disappearing after 90 days at room temperature.
580 Although our data cannot be compared with previous findings, *Saccharomyces* sporadic presence as
581 minor taxa in 103 SCOBY may suggest a minor role played in kombucha beverage (Harrison and
582 Curtin, 2021). Indeed, both *S. uvarum* and *S. cerevisiae* have been reported to occur as minor
583 species in black and green tea kombucha, respectively (Coton et al., 2017). However, changes in the
584 composition of yeast communities may be ascribed to different geographic, climatic and
585 environmental conditions encountered during fermentation and production processes (Mayser et al.,
586 1995; Teoh et al., 2004).

587 AAB isolates, identified by molecular methods, corresponded to *G. oxydans* (46%), *N. hansenii*
588 (36%) and *K. saccharivorans* (18%). Previous works, carried out using HTS analysis, reported that
589 the genus *Gluconacetobacter* was largely dominant (87-98% relative abundance) in different
590 kombucha samples originated from Canada, UK, Ireland and USA (Marsh et al., 2014). These
591 authors did not quote the genus *Komagataeibacter*, which was co-dominant in our kombucha
592 beverage, probably because bacterial databases were not yet updated to reflect the new
593 classification. Actually, *K. xylinus*, was recently reclassified from *Gluconacetobacter xylinus* (also

previously known as *Acetobacter xylinum*) (Yamada et al., 2012). However, the comparison with their data is difficult as the length of the 16S reads did not allow an accurate assignment beyond genus level, due to the high level of sequence homology, while our approach, utilising the isolation and molecular detection of taxa allowed the assignment to the species level. Other works, using both metabarcoding and culture-based methods, reported *G. oxydans* as the dominant species, consistently with our findings, followed by species of the genera *Acetobacter* and *Gluconacetobacter* (Coton et al., 2017; Reva et al., 2015). On the other hand, De Filippis at al. (2018), using culture-dependent and independent methods, reported that *G. saccharivorans* (now *K. saccharivorans*) and *G. xylinus* (now *K. xylinus*) accounted more than 90% of the bacterial microbiota, and Harrison and Curtin (2021), using high-throughput sequencing approaches, found that the major AAB taxa amongst 103 SCOBY belonged to *Komagataeibacter* (71%), *Acetobacter* (12%) and *Gluconobacter* (3.5%).

4.3. Effects of different storage times and temperatures on the microbiota of kombucha fermented beverage by culture independent methods

Data on the diversity of core yeast and bacterial communities obtained using culture-independent methods were consistent with those of culture-dependent analyses. Compared with massive sequencing, PCR-DGGE represents a suitable option to describe changes in yeast and AAB communities across storage times and temperatures.

Cluster analysis of yeast DGGE profiles detected important changes in yeast community composition in kombucha samples stored for 45 and 90 days at room temperature, compared with the other samples. *D. anomala* was highly represented across storage, even after 90 days at room temperature, while *Saccharomyces* species were strongly affected by storage temperature, as shown by their disappearance in samples stored for 45 and 90 days at room temperature. Such dynamics is supported by previous data on lambic beer, where *S. cerevisiae* was gradually outcompeted by *Dekkera/Brettanomyces*, after 4–8 months, when most short oligosaccharides were exhausted

620 (Bokulich et al., 2012; Steensels et al., 2015; Van Oevelen et al., 1977), possibly confirming the
621 ability of *D. anomala* to withstand the lack of nutrients (Steensels et al., 2015).

622 Cluster analysis of AAB confirmed not only data from culture-dependent methods, but also
623 the behaviour of yeast communities, as AAB species composition showed strong changes starting
624 from 20 days of storage, completely disappearing after 45 days at room temperature. Although
625 room temperature exerted a strong influence on AAB occurrence, when samples were stored at 4 °C
626 no changes in the AAB community composition was found, suggesting that long-term storage
627 should be performed at 4 °C, in order to maintain kombucha properties ascribed to AAB. It is
628 intriguing to speculate that the parallel disappearance of yeasts and AAB may be ascribed to the
629 strict metabolic relationship between these two microbial groups.

630 Cold storage could represent an important tool for long-term storage of kombucha. Indeed,
631 most commercially available kombucha drinks are pasteurized, in order to preserve their quality,
632 preventing alcohol production and acidification, and to prolong the shelf life, which may last up to
633 two years (Nummer, 2013). However, such process does not allow the maintenance of vitality of
634 putative probiotic strains, potentially lowering kombucha health-promoting properties.

635

636 *4.4. Qualitative functional characterization of yeast isolates*

637 In this work, 11 isolates of *D. anomala* and one of *S. uvarum* showed high ability to produce
638 organic acids “in vitro”, possibly contributing to pH lowering in kombucha beverages. Among the
639 58 yeast isolates, 29% of them were able to produce exopolysaccharides, belonging to *D. anomala*,
640 *Z. bailii, Z. parabailii* and *Z. lentus*. Moreover, three isolates of *D. anomala* showed a very high
641 activity. Such isolates could be further studied as potential starters for the production of innovative
642 and functional fermented foods (Palla et al. 2019; 2021). Further investigations aimed at assessing
643 their ability to survive into gastro-intestinal tracts could be of great interest in order to consider the
644 potential role of yeasts as probiotics. Indeed, as kombucha represents a stressful environment,
645 characterized by a low pH, as well as the digestive system, it is conceivable that yeasts isolated

646 from such peculiar conditions, may show abilities to survive gastrointestinal conditions (Palla et al.,
647 2021). Although so far most works performed on fermented foods have described bacterial
648 probiotic strains, recent studies reported the occurrence of probiotic traits in different yeast strains
649 belonging to *S. cerevisiae* isolated from sourdoughs of different origins and other fermented foods
650 (Palla et al., 2021; Perricone et al., 2014; Romanin et al., 2016; Şanlidere Aloğlu et al., 2016).
651 Indeed, a strain of *S. cerevisiae* var. *boulardii* is already on the market as a probiotic (Palma et al.,
652 2015).

653 Regarding protein hydrolysis, high protease activity was shown "in vitro" by two isolates of
654 *Z. bailii*, one of *Z. parabailii* and one of *Z. lentus*, while it was completely absent in all *D. anomala*
655 isolates. Proteolysis during kombucha fermentation provides amino acids whose catabolism may
656 benefit AAB. Indeed Tran et al. (2020b) reported that free amino nitrogen concentrations may
657 increase up to 54 μg/L during fermentation, as the result of yeast extracellular proteolytic activity
658 and/or autolysis.

659 The metabolic characteristics of the yeasts isolated in this work should be confirmed by
660 further quantitative studies, in order to exploit their potential as starters for the production of
661 functional kombucha beverages and other fermented foods and drinks, for example cereal-based
662 and not-dairy fermented beverages, utilized by vegans and lactose-intolerant consumers as an
663 alternative to fermented dairy products.

664

665 **5. Conclusion**

666 This was the first study monitoring the dynamics of yeasts and AAB across long term-storage (three
667 months) in a kombucha beverage. The study confirms that the combination of culture-dependent
668 and independent approaches is important for obtaining a more complete picture of the distinctive
669 core community of dominant yeasts and AAB in the beverage during storage and for elucidating its
670 diversity and composition. The use of culture-dependent methods allowed us to isolate and
671 preliminarily characterize yeasts and AAB strains, that could be further studied and functionally

characterised for their possible biotechnological implementation. Additional comparative studies are needed to understand how variations in microbial diversity, brewing process, fermentation time, herbs and fruits used for the infusion and storage time and temperature may affect the metabolic activities of yeasts and AAB and the quality of our kombucha beverage (Bishop et al., 2022; Tran et al., 2022a; b).

The kombucha beverage studied in this work represented not only a plant-based, not-dairy fermented beverage, but also a valuable source of potentially functional yeast strains. Further in-depth and comprehensive studies should be performed on the differential sensory and functional traits of our isolated strains, that might lead to the exploitation of their biotechnological potential and possible utilization as starters for the production of functional fermented foods, such as cereal-based fermented beverages.

CRediT authorship contribution statement

Monica Agnolucci, Manuela Giovannetti: conceptualization, supervision, writing original draft, funding acquisition. Arianna Grassi, Caterina Cristani, Michela Palla, Rosita Giorgi: investigation, methodology. Caterina Cristani, Michela Palla: formal analyses. Monica Agnolucci: writing – review and editing.

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Declaration of competing interest: none

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698

699 **References**

700 Agnolucci, M., Scarano, S., Santoro, S., Sassano, C., Toffanin, A., Nuti, M., 2007. Genetic and
701 phenotypic diversity of autochthonous *Saccharomyces* spp. strains associated to natural
702 fermentation of 'Malvasia delle Lipari'. Lett. Appl. Microbiol. 45, 657–662.
703 https://doi.org/10.1111/j.1472-765X.2007.02244.x.

704 Agnolucci, M., Tirelli, A., Cocolin, L., Toffanin, A., 2017. *Brettanomyces bruxellensis* yeasts:
705 Impact on wine and winemaking. World J. Microbiol. Biotechnol. 33, 180.
706 https://doi.org/10.1007/s11274-017-2345-z.

707 Andreson, M., Kazantseva, J., Kuldjärv, R., Malv, E., Vaikma, H., Kaleda, A., Kütt M-L., Vilu,
708 R., 2022. Characterisation of chemical, microbial and sensory profiles of commercial kombuchas.
709 Int. J. Food Microbiol. 373, 109715. https://doi.org/10.1016/j.ijfoodmicro.2022.109715

710 Arıkan, M., Mitchell, A.L., Finn, R.D., Gürel, F., 2020. Microbial composition of kombucha
711 determined using amplicon sequencing and shotgun metagenomics. J. Food Sci. 85, 455–464.
712 https://doi.org/10.1111/1750-3841.14992.

713 Bellut, K., Michel, M., Zarnkow, M., Hutzler, M., Jacob, F., De Schutter, D.P., Daenen, L.,
714 Zannini, E., Arendt, E.K., 2018. Application of non-*Saccharomyces* yeasts isolated from kombucha
715 in the production of alcohol-free beer. Fermentation 4, 66.
716 https://doi.org/10.3390/fermentation4030066.

717 Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers,
718 E.W., 2013. GenBank. Nucleic Acids Res. 41, D36–D42. https://doi.org/10.1093/nar/gks1195.

719 Bishop, P., Pitts, E.R., Budner, D., Thompson-Witrick, K.A., 2022. Kombucha: biochemical and
720 microbiological impacts on the chemical and flavor profile. Food Chem. Adv. 1, 100025.
721 https://doi.org/10.1016/j.focha.2022.100025.

722 Bokulich, N.A., Bamforth, C.W., Mills, D.A., 2012. Brewhouse-resident microbiota are
723 responsible for multi-stage fermentation of American coolship ale. PLoS One 7.
724 https://doi.org/10.1371/journal.pone.0035507.
725 Brandão, P.R., Crespo, M.T., Nascimento, F.X., 2022. Phylogenomic and comparative analyses
726 support the reclassification of several *Komagataeibacter* species as novel members of the
727 *Novacetimonas* gen. nov. and bring new insights into the evolution of cellulose synthase genes. Int.
728 J. Syst. Evol. Microbiol. 72, 005252. https://doi.org/10.1099/ijsem.0.005252.
729 Buron, N., Coton, M., Desmarais, C., Ledauphin, J., Guichard, H., Barillier, D., Coton, E., 2011.
730 Screening of representative cider yeasts and bacteria for volatile phenol-production ability. Food
731 Microbiol. 28, 1243–1251. https://doi.org/10.1016/j.fm.2011.05.001.
732 Buron, N., Coton, M., Legendre, P., Ledauphin, J., Kientz-Bouchart, V., Guichard, H., Barillier,
733 D., Coton, E., 2012. Implications of *Lactobacillus collinoides* and *Brettanomyces/Dekkera anomala*
734 in phenolic off-flavour defects of ciders. Int. J. Food Microbiol. 153, 159–165.
735 https://doi.org/10.1016/j.ijfoodmicro.2011.11.002.
736 Chakravorty, S., Bhattacharya, S., Chatzinotas, A., Chakraborty, W., Bhattacharya, D., Gachhui,
737 R., 2016. Kombucha tea fermentation: microbial and biochemical dynamics. Int. J. Food Microbiol.
738 220, 63–72. https://doi.org/10.1016/j. ijfoodmicro.2015.12.015.
739 Chen, C., Liu, B.Y., 2000. Changes in major components of tea fungus metabolites during
740 prolonged fermentation. J. Appl. Microbiol. 89, 834–839. https://doi.org/10.1046/j.1365-
741 2672.2000.01188.x.
742 Cocolin, L., Bisson, L.F., Mills, D.A., 2000. Direct profiling of the yeast dynamics in wine
743 fermentations. FEMS Microbiol. Lett. 189, 81–87. https://doi.org/10.1111/j.1574-
744 6968.2000.tb09210.x.
745 Coton, M., Pawtowski, A., Taminiau, B., Burgaud, G., Deniel, F., Coulloumme-Labarthe, L.,
746 Coton, E., 2017. Unraveling microbial ecology of industrial-scale kombucha fermentations by

metabarcoding and culture-based methods. FEMS Microbiol. Ecol. 93, 1–16. https://doi.org/10.1093/femsec/fix048.

De Filippis, F., Troise, A.D., Vitaglione, P., Ercolini, D., 2018. Different Temperatures Select Distinctive Acetic Acid Bacteria Species and Promotes Organic Acids Production during Kombucha Tea Fermentation. Food Microbiol. 73, 11–16. https://doi.org/10.1016/j.fm.2018.01.008.

Dufresne, C., Farnworth, E., 2000. Tea, kombucha and health: a review. Food Res. Int. 33, 409–421. https://doi.org/10.1016/S0963-9969(00)00067-3.

Diez-Ozaeta, I., Astiazaran, O.J., 2022. Recent advances in Kombucha tea: Microbial consortium, chemical parameters, health implications and biocellulose production. Int. J. Food Microbiol., 109783. https://doi.org/10.1016/j.ijfoodmicro.2022.109783

Domizio, P., Romani, C., Comitini, F., Gobbi, M., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Potential spoilage non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Ann Microbiol 61:137–144

Edgar, R.C., 2004a. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinf. 5, 1. https://doi.org/10.1186/1471-2105-5-113.

Edgar, R.C., 2004b. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797. https://doi.org/10.1093/nar/gkh340.

Egli, C.M., Henick-Kling, T., 2001. Identification of *Brettanomyces/Dekkera* species based on polymorphism in the rRNA internal transcribed spacer region. Am. J. Enol. Vitic. 52, 241–247.

Emiljanowicz, K.E., Malinowska-Pańczyk, E., 2020. Kombucha from alternative raw materials – the review. Crit. Rev. Food Sci. Nutr. 60, 3185–3194. https://doi.org/10.1080/10408398.2019.1679714,0.

Esteve-Zarzozo, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49, 329–337. https://doi.org/10.1099/00207713-49-1-329.

772 Fu, C., Yan, F., Cao, Z., Xie, F., Lin, J., 2014. Antioxidant activities of kombucha prepared from
773 three different substrates and changes in content of probiotics during storage. Food Sci. Technol.
774 (Zur.) 34, 123–126. https://doi.org/10.1590/S0101-20612014005000012.

775 Fernández-Espinar, M.T., Esteve-Zarzoso, B., Querol, A., Barrio, E., 2000. RFLP analysis of the
776 ribosomal internal transcribed spacers and the 5.8S rRNA gene region of the genus *Saccharomyces*:
777 a fast method for species identification and the differentiation of flor yeasts. Antonie Van
778 Leeuwenhoek 78, 87–97. https://doi.org/10.1023/a:1002741800609.

779 Gaggìa, F., Baffoni, L., Galiano, M., Nielsen, D.S., Jakobsen, R.R., Castro-Mejía, J.L., Bosi, S.,
780 Truzzi, F., Musumeci, F., Dinelli, G., Di Gioia, D., 2019. Kombucha beverage from green, black
781 and rooibos teas: a comparative study looking at microbiology, chemistry and antioxidant activity.
782 Nutrients 11, 1–22. https://doi.org/10.3390/nu11010001.

783 Garavaglia, J., Schneider, R. de C. de S., Camargo Mendes, S.D., Welke, J.E., Zini, C.A.,
784 Caramão, E.B., Valente, P., 2015. Evaluation of *Zygosaccharomyces bailii* BCV 08 as a co-starter
785 in wine fermentation for the improvement of ethyl esters production. Microbiol. Res. 173, 59–65.
786 https://doi.org/10.1016/j.micres.2015.02.002.

787 Garofalo, C., Osimani, A., Milanović, V., Aquilanti, L., De Filippis, F., Stellato, G., Di Mauro,
788 S., Turchetti, B., Buzzini, P., Ercolini, D., Clementi, F., 2015. Bacteria and yeast microbiota in milk
789 kefir grains from different Italian regions. Food microbiol. 49, 123–133.
790 https://doi.org/10.1016/j.fm.2015.01.017.

791 Greenwalt, C.J., Steinkraus, K.H., Ledford, R.A., 2000. Kombucha, the fermented tea:
792 microbiology, composition, and claimed health effects. J. Food Prot. 63, 976–981.
793 https://doi.org/10.4315/0362-028X-63.7.976.

794 Greenwalt, C.J., Ledford, R.A., Steinkraus, K.H., 1998. Determination and characterization of
795 the antimicrobial activity of the fermented tea kombucha. Lebensm. Wiss. Technol. 31, 291–296.
796 https://doi.org/10.1006/fstl.1997.0354.
797 Gullo, M., La China, S., Falcone, P.M., and Giudici, P., 2018. Biotechnological production of
798 cellulose by acetic acid bacteria: current state and perspectives. Appl. Microbiol. Biotechnol. 102,
799 6885–6898. https:// doi.org/10.1007/s00253-018-9164-5.
800 Harrison, K., Curtin, C., 2021. Microbial composition of SCOBY starter cultures used by
801 commercial kombucha brewers in North America. Microorganisms, 9, 1060.
802 https://doi.org/10.3390/microorganisms9051060.
803 Hrnjez, D., Vaštag, Ž., Milanovic, S., Vukic, V., Ilicic, M., Popovic, L.J., Kanuric, K., 2014. The
804 biological activity of fermented dairy products obtained by kombucha and conventional starter
805 cultures during storage. J. Funct. Foods 10, 336–345. https://doi.org/10.1016/j.jff.2014.06.016.
806 Jafari, R., Naghavi, N.S., Khosravi-Darani, K., Doudi, M., Shahanipour, K., 2021. Isolation,
807 molecular and phylogenetic identification of microorganisms from kombucha solution and
808 evaluation of their viability using flow cytometery. Food Sci. Technol, Campinas, v42, e63220.
809 https://doi.org/10.1590/fst.63220.
810 Jayabalan, R., Malbaša, R.V., Lončar, E.S., Vitas, J.S., Sathishkumar, M., 2014. A review on
811 Kombucha tea—microbiology, composition, fermentation, beneficial effects, toxicity, and tea
812 fungus. Compr. Rev. Food Sci. Food Saf. 13, 538–550. https://doi.org/10.1111/1541-4337.12073.
813 Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In Munro HN,
814 editor, Mammalian Protein Metabolism, pp. 21–132, Academic Press, New York.
815 Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions
816 through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
817 https://doi.org/10.1007/BF01731581.

818 Kurtzman, C.P., Robnett, C.J., 1998. Identification and phylogeny of ascomycetous yeasts from
819 analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van
820 Leeuwenhoek 73, 331–371. https://doi.org/10.1023/a:1001761008817.
821 La Torre, C., Fazio, A., Caputo, P., Plastina, P., Caroleo, M.C., Cannataro, R., Cione, E., 2021.
822 Effects of Long-Term Storage on Radical Scavenging Properties and Phenolic Content of
823 Kombucha from Black Tea. Molecules 26, 5474. https://doi.org/10.3390/molecules26185474
824 Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.),
825 Nucleic Acid Techniques in Bacterial Systematics. Wiley, New York, pp. 115–175.
826 Laureys, D., Britton, S.J., De Clippeleer, J., 2020. Kombucha tea fermentation: a review. J. of
827 Am. Soc. Brew. Chem. 78, 165–174. https://doi.org/10.1080/03610470.2020.1734150.
828 Leonarski, E., Cesca, K., Zanella, E., Stambuk, B.U., de Oliveira, D., Poletto, P., 2021.
829 Production of kombucha beverage and bacterial cellulose by acerola by product as raw material.
830 LWT 135, 110075. https://doi.org/10.1016/j.lwt.2020.110075.
831 Li, J., Hu, W., Huang, X., Xu, Y., 2018. Investigation of yeast population diversity and dynamics
832 in spontaneous fermentation of Vidal blanc icewine by traditional culture-dependent and high-
833 throughput sequencing methods. Food Res. Int. 112, 66–77.
834 https://doi.org/10.1016/j.foodres.2018.06.011.
835 Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., VanderGheynst, J.,
836 Mills, D.A., 2003. Design and evaluation of PCR primers for analysis of bacterial populations in
837 wine by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 69, 6801–6807.
838 https://doi.org/10.1128/AEM.69.11.6801-6807.2003.
839 Marsh, A.J., Sullivan, O.O., Hill, C., Ross, R.P., Cotter, P.D., 2014. Sequence-based analysis of
840 the bacterial and fungal compositions of multiple Kombucha (tea fungus) samples. Food Microbiol.
841 38, 171–178. https://doi.org/10.1016/j.fm.2013.09.003.
842 Mayser, P., Gromme, S., Leitzmann, C., Grunder, K., 1995. The yeast spectrum of the ‘tea
843 fungus Kombucha’. Mycoses 38, 289 – 295. https://doi.org/10.1111/j.1439-0507.1995.tb00410.x.

844 Neffe-Skocińska, K., Sionek, B., Ścibisz, I., Kołożyn-Krajewska, D., 2017. Acid contents and
845 the effect of fermentation condition of kombucha tea beverages on physicochemical,
846 microbiological and sensory properties. CyTA - J. Food 15, 601–607.
847 https://doi.org/10.1080/19476337.2017.1321588.
848 Nummer, B.A., 2013. Kombucha brewing under the food and drug administration model Food
849 Code: Risk analysis and processing guidance. (Special Report). J. Environ. Health 76, 8–1.
850 Nyhan, L.M., Lynch, K.M., Sahin, A.W., Arendt, E.K., 2022. Advances in Kombucha Tea
851 Fermentation: A Review. Appl. Microbiol. 2, 73–103.
852 https://doi.org/10.3390/applmicrobiol2010005
853 Palla, M., Cristani, C., Giovannetti, M., Agnolucci, M., 2017. Identification and characterization
854 of lactic acid bacteria and yeasts of PDO Tuscan bread sourdough by culture dependent and
855 independent methods. Int. J. Food Microbiol. 250, 19–26.
856 https://doi.org/10.1016/j.ijfoodmicro.2017.03.015.
857 Palla, M., Digiacomo, M., Cristani, C., Bertini, S., Giovannetti, M., Macchia, M., Manera, C.,
858 Agnolucci, M., 2018. Composition of health-promoting phenolic compounds in two extra virgin
859 olive oils and diversity of associated yeasts. J. Food Compost. Anal. 74, 27–33.
860 https://doi.org/10.1016/j.jfca.2018.08.008.
861 Palla, M., Agnolucci, M., Calzone, A., Giovannetti, M., Di Cagno, R., Gobbetti, M., ... &
862 Pontonio, E. (2019). Exploitation of autochthonous Tuscan sourdough yeasts as potential starters.
863 Int. J. Food Microbiol. 302, 59–68. https://doi.org/10.1016/j.ijfoodmicro.2018.08.004.
864 Palla, M., Blandino, M., Grassi, A., Giordano, D., Sgherri, C., Quartacci, M.F., Reyneri, A.,
865 Agnolucci, M., Giovannetti, M., 2020. Characterization and selection of functional yeast strains
866 during sourdough fermentation of different cereal wholegrain flours. Sci. Rep. 10, 1–15.
867 https://doi.org/ 10.1038/s41598-020-69774-6.

Palla, M., Conte, G., Grassi, A., Esin, S., Serra, A., Mele, M., Giovannetti, M., Agnolucci, M., 2021. Novel Yeasts Producing High Levels of Conjugated Linoleic Acid and Organic Acids in Fermented Doughs. Foods 10, 2087. https://doi.org/10.3390/foods10092087.

Pallmann, C.L., Brown, J.A., Olineka, T.L., Cocolin, L., Mills, D.A., Bisson, L.F., 2001. Use of WL medium to profile native flora fermentations. Am. J. Enol. Vitic. 52, 198–203.

Palma, M.L., Zamith-Miranda, D., Martins, F.S., Bozza, F.A., Nimrichter, L., Montero-Lomeli, M., Marques, E.T., Douradinha, B., 2015. Probiotic *Saccharomyces cerevisiae* strains as biotherapeutic tools: is there room for improvement? Appl. Microbiol. Biotechnol. 99, 6563–6570. https://doi.org/10.1007/s00253-015-6776-x.

Perricone, M., Bevilacqua, A., Corbo, M.R., Sinigaglia, M., 2014. Technological characterization and probiotic traits of yeasts isolated from Altamura sourdough to select promising microorganisms as functional starter cultures for cereal-based products. Food Microbiol. 38, 26–35. https://doi.org/10.1016/j.fm.2013.08.006.

Rai, A.K., Pandey, A., Sahoo, D., 2019. Biotechnological potential of yeasts in functional food industry. Trends Food Sci. Technol. 83, 129–137. https://doi.org/10.1016/j.tifs. 2018.11.016.

Reva, O.N., Zaets, I.E., Ovcharenko, L.P., Kukharenko, O.E., Shpylova, S.P., Podolich, O.V, de Vera, J-P., Kozyrovska, N.O., 2015. Metabarcoding of the kombucha microbial community grown in different microenvironments. AMB Express 5, 1–8. https://doi.org/10.1186/s13568-015-0124-5.

Ricciardi, A., Parente, E., Clementi, F., 1997. A simple method for the screening of lactic acid bacteria for the production of exopolysaccharides in liquid media. Biotechnol. Tech. 11, 271–275.

Romanin, D.E., Llopis, S., Genoves, S., Martorell, P., Ramón, V., Garrote, G.L., Rumbo, M., 2016. Probiotic yeast *Kluyveromyces marxianus* CIDCA 8154 shows anti-inflammatory and anti-oxidative stress properties in in vivo models. Benef. Microbes 7, 83–93. https://doi.org/10.3920/BM2015.0066.

892 Sampaio, J.P., Pontes, A., Libkind, D., Hutzler, M., 2017. Taxonomy, diversity, and typing of
893 brewing yeasts. In: Bokulich, N.A., Bamforth, C.W. (Eds.), Brewing Microbiology. Caister
894 Academic Press, Norfolk, UK, pp. 85–117. https://doi.org/10.21775/9781910190616.04.
895 Şanlidere Aloğlu, H., Demir Özer, E., Öner, Z., 2016. Assimilation of cholesterol and probiotic
896 characterisation of yeast strains isolated from raw milk and fermented foods. Int. J. Dairy Technol.
897 69, 63–70. https://doi.org/10.1111/1471-0307.12217.
898 Sarkaya, P., Akan, E., Kinik, O., 2021. Use of kombucha culture in the production of fermented
899 dairy beverages. LWT 137, 110326. https://doi.org/10.1016/j.lwt.2020.110326.
900 Smith, B.D., Divol, B., 2016. *Brettanomyces bruxellensis*, a survivalist prepared for the wine
901 apocalypse and other beverages. Food Microbiol. 59, 161–175.
902 https://doi.org/10.1016/j.fm.2016.06.008.
903 Steensels, J., Daenen, L., Malcorps, P., Derdelinckx, G., Verachtert, H., Verstrepen, K.J., 2015.
904 *Brettanomyces* yeasts - from spoilage organisms to valuable contributors to industrial fermentations.
905 Int. J. Food Microbiol. 206, 24–38. https://doi.org/10.1016/j.ijfoodmicro.2015.04.005.
906 Suh, S.O., Gujjari, P., Beres, C., Beck, B., Zhou, J., 2013. Proposal of *Zygosaccharomyces*
907 *parabailii* sp. nov. and *Zygosaccharomyces pseudobailii* sp. nov., novel species closely related to
908 *Zygosaccharomyces bailii*. Int. J. Syst. Evol. Microbiol. 63, 1922–1929.
909 Tamura, K., Stecher, G., Kumar, S., 2021. MEGA 11: Molecular Evolutionary Genetics Analysis
910 Version 11. Molecular Biology and Evolution. https://doi.org/10.1093/molbev/msab120.
911 Tan, W.C., Muhialdin, B.J., Meor Hussin, A.S., 2020. Influence of Storage Conditions on the
912 Quality, Metabolites, and Biological Activity of Soursop (*Annona muricata* L.) Kombucha. Front.
913 Microbiol. 11, 2982. https://doi.org/10.3389/fmicb.2020.603481.
914 Teoh, A.L., Heard, G., Cox, J., 2004. Yeast ecology of Kombucha fermentation. Int. J. Food
915 Microbiol. 95, 119–126. https://doi.org/10.1016/j.ijfoodmicro.2003.12.020.
916 Thomas, D.S., Davenport, R.R., 1985. *Zygosaccharomyces bailii* - a profile of characteristics and
917 spoilage activities. Food Microbiol. 2, 157–169. https://doi.org/10.1016/S0740-0020(85)80008-3.

Tran, T., Grandvalet, C., Verdier, F., Martin, A., Alexandre, H., Tourdot-Maréchal, R., 2020a. Microbiological and technological parameters impacting the chemical composition and sensory quality of kombucha. Compr. Rev. Food Sci. Food Saf. 19, 2050-2070. https://doi.org/10.1111/1541-4337.12574.

Tran, T., Grandvalet, C., Verdier, F., Martin, A., Alexandre, H., Tourdot-Maréchal, R., 2020b. Microbial dynamics between yeasts and acetic acid bacteria in kombucha: impacts on the chemical composition of the beverage. Foods 9, 963. https://doi.org/10.3390/foods9070963.

Tran, T., Grandvalet, C., Winckler, P., Verdier, F., Martin, A., Alexandre, H., Tourdot-Maréchal, R., 2021. Shedding light on the formation and structure of kombucha biofilm using two-photon fluorescence microscopy. Front. Microbiol. 2204. https://doi.org/10.3389/fmicb.2021.725379.

Tran, T., Billet, K., Torres-Cobos, B., Vichi, S., Alexandre, H., Grandvalet, C., Tourdot-Maréchal, R., 2022a. Use of a minimal microbial consortium to determine the origin of kombucha flavor. Front. Microbiol. 13, 836617. https://doi.org/10.3389/fmicb.2022.836617.

Tran, T., Roullier-Gall, C., Verdier, F., Martin, A., Schmitt-Kopplin, P., Alexandre, H., Grandvalet, C., Tourdot-Maréchal, R., 2022b. Microbial interactions in kombucha through the lens of metabolomics. Metabolites 12, 235. https://doi.org/10.3390/metabo12030235.

Van Oevelen, D., Spaepen, M., Timmermans, P., Verachtert, H., 1977. Microbiological as- pects of spontaneous wort fermentation in the production of lambic and gueuze. J. Inst. Brew. 83, 356–360. https://doi.org/10.1002/j.2050-0416.1977.tb03825.x.

Wang, B., Rutherfurd-Markwick, K., Zhang, X.X., Mutukumira, A.N., 2022. Isolation and characterisation of dominant acetic acid bacteria and yeast isolated from kombucha samples at point of sale in New Zealand. Curr. Res. Food Sci. 5, 835–844. https://doi.org/10.1016/j.crfs.2022.04.013.

Watawana, M., N. Jayawardena, C. Gunawardhana, Waisundara, V., 2015. Health, wellness, and safety aspects of the consumption of kombucha. J. Chem. 1–11. https://doi.org/10.1155/2015/591869.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: a Guide to Methods and Applications. Academic Press Inc., New York, pp. 315–322.

Yamada, Y., Yukphan, P., Vu, H.T.L., Muramatsu, Y., Ochaikul, D., Tanasupawat, S., Nakagawa, Y., 2012. Description of *Komagataeibacter* gen. nov., with proposals of new combinations (*Acetobacteraceae*). J. Gen. Appl. Microbiol. 58, 397–404. https://doi.org/10.2323/jgam.58.397.

Zagrabinski, T., 2020. History of Kombucha - Part 1. Baerbucha kombucha. Available online https://www.baerbucha-kombucha.com/post/history-of-kombucha-part-1 [Accessed August 29, 2022]

Zotta, T., Giavalisco, M., Parente, E., Picariello, G., Siano, F., Ricciardi, A., 2022. Selection of *Lactiplantibacillus* strains for the production of fermented table olives. Microorganisms 10, 625. https://doi.org/10.3390/microorganisms10030625.

Tables

Table 1 Microbiological analyses of the kombucha fermented beverage stored at 4 °C and at room temperature for a period of 90 days.

		T0	T3			T20			T45			T90		
Microbial group[a]	Media		25 °C	4 °C		25 °C	4 °C		25 °C	4 °C		25 °C	4 °C	
Total mesophilic aerobic bacteria	PCA	6.85 ± 0.04	6.78 ± 0.02	6.78 ± 0.07		5.31 ± 0.16	5.45 ± 0.13		**2.39 ± 0.07**	**5.14 ± 0.06**	***	**1.45 ± 0.04**	**5.37 ± 0.15**	*
Yeasts	WL	6.78 ± 0.06	6.66 ± 0.08	6.77 ± 0.01		6.90 ± 0.10	6.96 ± 0.07		6.70 ± 0.11	6.68 ± 0.02		6.39 ± 0.29	6.84 ± 0.01	
	SDA	6.84 ± 0.09	6.72 ± 0.02	6.73 ± 0.06		6.87 ± 0.01	7.00 ± 0.02		6.56 ± 0.04	6.66 ± 0.04		6.26 ± 0.50	6.73 ± 0.05	
FGY	WL	5.57 ± 0.05	5.42 ± 0.06	5.35 ± 0.06		5.18 ± 0.30	5.48 ± 0.39		**2.95 ± 0.21**	**5.09 ± 0.08**	*	**n.d.**	**5.35 ± 0.12**	*
	SDA	5.65 ± 0.07	5.35 ± 0.10	5.27 ± 0.02		5.36 ± 0.27	5.52 ± 0.14		2.65 ± 0.56	5.06 ± 0.12		**n.d.**	**5.24 ± 0.28**	*
SGY	WL	6.75 ± 0.06	6.64 ± 0.08	6.75 ± 0.01		6.89 ± 0.11	6.94 ± 0.06		6.70 ± 0.11	6.67 ± 0.02		6.39 ± 0.29	6.83 ± 0.01	
	SDA	6.81 ± 0.09	6.70 ± 0.02	6.71 ± 0.06		6.86 ± 0.02	6.99 ± 0.03		6.56 ± 0.04	6.65 ± 0.04		6.26 ± 0.50	6.72 ± 0.04	
AAB	WL	4.66 ± 0.08	4.14 ± 0.05	4.25 ± 0.04		3.46 ± 0.09	4.48 ± 0.39		**1.53 ± 0.23**	**3.90 ± 0.10**	*	**n.d.**	**3.87 ± 0.04**	**
	YPM	4.47 ± 0.10	4.11 ± 0.02	4.17 ± 0.00		3.31 ± 0.05	4.37 ± 0.37		**0.52 ± 0.00**	**3.45 ± 0.16**	*	**n.d.**	**3.73 ± 0.21**	*
LAB	MRS	2.30 ± 0.14	**n.d.**	**1.45 ± 0.04**	*	n.d.	n.d.		n.d.	n.d.		n.d.	n.d.	

Values indicate mean Log CFU/mL ± standard deviations (SD) among three replicates per sample. N.d.: not detectable.

Means on the same line and within the same storage period followed by asterisk are significantly different (***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$).
[a] FGY: fast-growing yeasts (*Saccharomyces* spp.); SGY: slow-growing yeasts (*D. anomala* and *Zygosaccharomyces* spp.); AAB: Acetic Acid Bacteria; LAB: Lactic Acid Bacteria.

[b] PCA: Plate Count Agar; WL: Wallerstein Laboratory Nutrient agar; SDA: Sabouraud Dextrose Agar; YPM: Yeast Peptone Mannitol agar; MRS: Man Rogosa Sharpe agar.

Table 2 Differentiation of the 58 yeasts, isolated from the kombucha fermented beverage, according to their growth rate and genetic characterization (ITS amplicons, ITS-RFLP patterns and species-specific PCR). Isolates in bold were identified through sequencing of D1/D2 region of 26S rRNA gene.

Isolates	Growth rate	Species-specific PCR for *D. anomala*	ITS sizes	ITS-RFLP patterns		Presumptive species
				***Hae*III**	***Hpa*II**	
IMA **K8Y**, K26Y, K28Y, K43Y, K46Y, KRY	Fast	_	850 bp	325, 230, 170, 125 bp	725, 125 bp	*S. cerevisiae*
IMA K25Y, K29Y, K35Y, K42Y, K44Y, K47Y				495, 230, 125 bp	725, 125 bp	*S. bayanus/pastorianus*
IMA K1Y, K20Y, K24Y, K30Y, K49Y, KCY, KEY				495, 230, 125 (325, 170) bp*	725, 125 bp	*S. bayanus/pastorianus*
IMA K23Y, **K31Y**, **K32Y**, **K40Y**, **K41Y, K45Y**, **KQY**	Slow	No amplicons	780 bp	–	–	–
IMA K36Y, K48Y			700 bp			–
IMA K2Y-K7Y, K10Y-K19Y, K21, K22, K27Y, K33Y, K34Y, K37Y-K39Y, **KDY**, KFY, KHY, KNY, KOY, KPY, *D. anomala* ATCC 10562		450 bp	510 bp			*D. anomala*

*This profile encompasses two weak additional fragments (see paragraph 3.1.2.).

Table 3 Best-match identification of yeasts and acetic acid bacteria (AAB) isolates and the DGGE fragments, as obtained by nBLAST.

	TAXON NAME	CLOSEST MATCH (% similarity*)	GENBANK ACCESSION N.
AAB ISOLATE			
KB	*G. oxydans* DSM 3504	99.85	CP004373
KG	*G. oxydans* DS5MA	100	LN884063
K1	*G. oxydans* DSM 3504	100	CP004373.1
K5	*G. oxydans* DSM 3504	99.78	CP004373.1
K17	*N. hansenii* NBRC 14820	99.93	NR_113674.1
K23	*N. hansenii* NBRC 14820	99.93	NR_113674.1
K28	*N. hansenii* KGB	100	LT546164.1
K31	*G. oxydans* DSM 3504	99.93	CP004373.1
K52	*N. hansenii* NBRC 14820	100	NR_112227.1
K62	*K. saccharivorans* JCM 25121	99.93	NR_113398.1
K66	*K. saccharivorans* JCM 25121	99.71	NR_113398.1
AAB DGGE FRAGMENT			
1	*G. oxydans* Go1/*G. cerevisiae* LMG27749/*G. aidae* AC10	99.68	MN909110.1/HG424633.1/LC511690.1
3	*K. saccharivorans* JCM 25121/*K. xylinus* CGMCC 17276/*K. diospyri* MSKU	99.09	NR_113398.1/CP041348.1/MG971339.2
5	*K. oboediens* LTH2460/*K. rhaeticus* DSM 16663/*K. melomenusus* strain AV436	99.69	NR_114683.1/NR_118187.1/MT422127.1
6	*N. hansenii* DSM 5602/*Ga. entanii* LTH4560	100	NR_118178.1/NR_028909.1
9	*K. saccharivorans* LMG 1582/*K. xylinus* XJL-06-4	99.37	NR_118189.1/MH447173.1
10	*K. medellinensis* NBRC 3288/*K. intermedius* TF2	99.69	NR_125626.1/NR_026435.1
13	*G. oxydans* Go1/*G. cerevisiae* LMG27749/*G. aidae* AC10	99.68	MN909110.1/HG424633.1/LC511690.1
14	*G. oxydans* Go1/*G. cerevisiae* LMG27749/*G. aidae* AC10	99.68	MN909110.1/HG424633.1/LC511690.1
15	*K melaceti* AV382/*K. europaeus* DHBR3702	99.37	MT422125.1/MH845618.1
16	*K. intermedius* JCM 16936/*K. medellinensis* NBRC 3288	99.69	NR_113394.1/NR_074338.1
18	*N. hansenii* strain Gachhui RG3/*Ga. entanii* strain LTH4560	99.69	NR_115108.1/NR_028909.1
20	*G. oxydans* Go1/*G. cerevisiae* LMG27749/*G. aidae* AC10	99.68	MN909110.1/HG424633.1/LC511690.1
21	*K. oboediens* strain LTH2460/*K. rhaeticus* strain JCM 17122	99.06	NR_114683.1/NR_113396.1
23	*K. oboediens* LTH2460/*K. rhaeticus* JCM 17122	99.69	NR_114683.1/NR_113396.1
YEAST ISOLATE			
K8	*S. cerevisiae* NRRL Y-12632	99.49	NG_042623.1
K1	*S. uvarum* VA12	100	LT009475.1
K20	*S. uvarum* SF5-310-4II8	99.83	MH041895.1
K24	*S. uvarum* SF5-310-4II8	99.65	MH041895.1
K25	*S. uvarum* VA12	100	LT009475.1
K29	*S. uvarum* DBVPG 4171	99.82	EU020102.1
K30	*S. uvarum* SF5-310-4II8/*S.* cf. *bayanus*/*pastorianus* CBS:2442	100	MH041895.1/KY109231.1
K35	*S. uvarum* CBS:10272/*S. bayanus* NRRL Y-12624/*S.* cf. *bayanus/pastorianus* CBS:2898	99.84	KY109469.1/NG_055690.1/ KY109228.1
K42	*S. uvarum* SF5-310-4II8	100	MH041895.1
K44	*S. uvarum* CBS:8712/*S. bayanus* CBS:8690/*S.*	99.67	KY109467.1/KY109231.1/KY109228.1

	bayanus/pastorianus CBS:2898		
K47	*S. uvarum* SF5-310-4II8/*S. bayanus* CBS:8690/*S. bayanus/pastorianus* CBS:2898	100	MH041895.1/ KY109213.1/ KY109228.1
K49	*S. uvarum* CBS:10272/*S. bayanus* CBS:8690/*S. bayanus/pastorianus* CBS:2898	100	KY109469.1/ KY109213.1/ KY109228.1
KC	*S. uvarum* CBS:10272/*S. bayanus* CBS:8690/*S.* cf. *bayanus/pastorianus* CBS:2898	99.83	KY109469.1/ KY109231.1/ KY109228.1
KE	*S. uvarum* CBS:10272/*S. bayanus* CBS:8690/*S.* cf. *bayanus/pastorianus* CBS:2898	100	KY109469.1/ KY109231.1/ KY109228.1
K23	*Z. parabailii* ATCC 60483	99.83	CP019493.1
K31	*Z. bailii* CBS:7555	99.63	KY110233.1
K32	*Z. bailii* ATCC 58445	100	NG_055054.1
K40	*Z. parabailii* ATCC 60483	99.51	MH930858.1
K41	*Z. bailii* ATCC 58445	99.83	NG_055054.1
K45	*Z. parabailii* ATCC 60483	99.84	CP019493.1
KQ	*Z. parabailii* ATCC 60483	99.84	CP019493.1
K36	*Z. lentus* CBS 8574	100	NG_058448.1
K48	*Z. lentus* CBS 8574	100	NG_058448.1
KD	*D. anomala* CBS 4711	100	AY969092.1
YEAST DGGE FRAGMENT			
2	*S. uvarum* CBS:426/*S. eubayanus* CBS 12357/*S. pastorianus* CBS:1503/*S. eubayanus* x *S. uvarum* CBS:1505/*S. bayanus* CBS:8697	99.18	KY109472.1/CP030956.1/KY109459.1/KY109433.1/KY109214.1
3	*S. uvarum* CBS:426/*S. eubayanus* CBS 12357/*S. pastorianus* CBS:1503/*S. eubayanus* x *S. uvarum* CBS:1505/*S. bayanus* CBS:8697	99.51	KY109472.1/CP030956.1/KY109459.1/KY109433.1/KY109214.1
4	*Z. bailii* culture CBS:4691/*Z. parabailii* ATCC 60483/*Z. pseudobailii* ATCC 56074	98.78	KY110241.1/CP019493.1/JQ745267.1
8	*D. anomala* strain DSMZ 70732	99.18	DQ406714.1
9	*D. anomala CBS:76*	97.42	KY107592.1
10	D. *anomala* CBS:4461	99.59	KY107595.1
13	*S. paradoxus* CBS:10267/*S. cerevisiae* isolate 0S3	95.00/94.53	KY109447.1/KP070747.1
14	*S. cerevisiae* HBUAS61172	98.56	MZ853707.1
16	*D. anomala* CBS:4461	96.72	KY107595.1
17	*D. anomala* CBS:4461	99.59	KY107595.1
18	*D. anomala* CBS: 4461	98.54	KY107595.1
19	*D. anomala* DSMZ	96.08	KY107595.1
20	*D. anomala* strain DSMZ 70732	99.59	DQ406714.1
22	*Z. lentus CBS 8574*	100	NG_058448.1
24	*S. uvarum* CBS:426/*S. eubayanus* CBS 12357/*S. pastorianus* CBS:1503/*S. eubayanus* x *S. uvarum* CBS:1505/*S. bayanus* CBS:8697	99.18	KY109472.1/CP030956.1/KY109459.1/KY109433.1/KY109214.1
25	*S. uvarum* CBS:426/*S. eubayanus* CBS 12357/*S. pastorianus* CBS:1503/*S. eubayanus* x *S. uvarum* CBS:1505/*S. bayanus* CBS:8697	99.50	KY109472.1/CP030956.1/KY109459.1/KY109433.1/KY109214.1
36	*S. cerevisiae* 0S3	95.72	KP070747.1
37	*S. cerevisiae* IUVV:VAlMiS V34OC1.2	99.60	MH276974.1
39	*D. anomala* DSMZ 70732	98.76	DQ406714.1

* Similarity represents the % similarity shared with the sequences in the GenBank database

Table 4 *In vitro* screening of organic acids, exopolysaccharides (EPS) production and protease activity of 58 yeasts isolated from kombucha fermented beverage. For organic acids production and protease activity, values indicate mean halo zone (mm) ± standard error (SE). EPS production: "-" = no production; "high = high production; "very high" = very high production.

Isolates	Organic acids production	EPS production[a]	Protease activity
D. anomala IMA K3Y	4.67±0.44	high	-
D. anomala IMA K4Y	6.67±0.93	-	-
D. anomala IMA K5Y	3.00±0.50	high	-
D. anomala IMA K6Y	6.00±0.29	high	-
D. anomala IMA K7Y	6.00±0.29	-	-
D. anomala IMA K2Y	5.17±0.17	-	-
D. anomala IMA K10Y	6.67±0.17	-	-
D. anomala IMA K11Y	7.50±0.58	-	-
D. anomala IMA K12Y	6.83±0.17	-	-
D. anomala IMA K13Y	7.17±0.67	high	-
D. anomala IMA K14Y	4.00±0	high	-
D. anomala IMA K15Y	6.42±0.08	-	-
D. anomala IMA K16Y	7.00±0.29	high	-
D. anomala IMA K17Y	5.33±0.60	-	-
D. anomala IMA K18Y	6.67±0.60	-	-
D. anomala IMA K19Y	7.00±0.29	-	-
D. anomala IMA K21Y	5.33±0.33	-	-
D. anomala IMA K22Y	7.83±0.33	-	-
D. anomala IMA K27Y	7.83±0.17	very high	-
D. anomala IMA K33Y	6.17±0.88	high	-
D. anomala IMA K34Y	6.67±0.17	-	-
D. anomala IMA K37Y	7.50±0.76	very high	-
D. anomala IMA K38Y	6.67±0.44	-	-
D. anomala IMA K39Y	7.17±0.44	-	-
D. anomala IMA KDY	7.42±0.08	-	-
D. anomala IMA KFY	7.00±0.58	-	-
D. anomala IMA KHY	6.00±0.50	high	-
D. anomala IMA KNY	7.50±0.50	high	-
D. anomala IMA KOY	6.00±0.29	high	-
D. anomala IMA KPY	6.00±1.00	very high	-
S. cerevisiae IMA K8Y	6.08±0.42	-	2.17±0.17
S. cerevisiae IMA K26Y	6.33±0.17	-	2.33±0.17
S. cerevisiae IMA K28Y	5.67±0.17	-	4.17±0.17
S. cerevisiae IMA K42Y	6.33±0.33	-	3.58±0.30
S. cerevisiae IMA K43Y	5.00±0.29	-	2.67±0.33
S. cerevisiae IMA K46Y	5.33±0.60	-	4.67±0.73
S. cerevisiae IMA KRY	4.33±0.17	-	1.50±0.29
S. uvarum IMA K1Y	5.33±0.33	-	5.08±0.08
S. uvarum IMA K20Y	7.33±0.33	-	2.50±0.29
S. uvarum IMA K24Y	6.33±0.44	-	4.00±0.58
S. uvarum IMA K25Y	5.83±0.88	-	3.00±0.58
S. uvarum IMA K29Y	5.67±0.33	-	4.17±0.17
S. uvarum/S. bayanus/S. bayanus/pastorianus IMA K30Y	5.00±0	-	3.92±0.08
S. uvarum/S. bayanus/S. bayanus/pastorianus IMA K35Y	6.50±0.29	-	3.67±0.17

S. uvarum/S. bayanus/S. bayanus/pastorianus IMA K44Y	6.17±0.44	-	2.67±0.33
S. uvarum/S. bayanus/S. bayanus/pastorianus IMA K47Y	6.83±0.44	-	2.33±0.33
S. uvarum/S. bayanus/S. bayanus/pastorianus IMA K49Y	6.50±0.50	-	-
S. uvarum/S. bayanus/S. bayanus/pastorianus IMA KCY	4.92±0.58	-	4.00±0.29
S. uvarum/S. bayanus/S. bayanus/pastorianus IMA KEY	4.75±0.38	-	3.67±0.33
Z. parabailii IMA K23Y	4.50±0.29	high	4.50±0
Z. bailii IMA K31Y	5.17±0.17	-	5.50±0.58
Z. bailii IMA K32Y	6.00±0.29	-	6.83±0.17
Z. parabailii IMA K40Y	6.67±0.17	high	6.17±0.93
Z. bailii IMA K41Y	3.67±0.33	high	8.58±1.75
Z. parabailii IMA K45Y	5.83±0.44	-	3.67±0.17
Z. parabailii IMA KQY	6.00±0.29	-	2.67±0.17
Z. lentus IMA K36Y	4.50±0.29	high	3.00±0.29
Z. lentus IMA K48Y	5.50±0.29	-	8.08±0.22

FIGURE CAPTIONS

Fig. 1. Light microscopy images showing **a:** ellipsoid cells of *Saccharomyces* species and **b:** ogival cells of *Dekkera anomala*.

Fig. 2. Affiliation of the sequences of the yeasts isolated from samples of the kombucha fermented beverage with the existing sequences of the D1/D2 region of the large sub-unit rRNA gene. Phylogenetic analysis was inferred by using the Maximum Likelihood method based on the kimura 2-parameter model. Bootstrap (1000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA11. The sequences from the database are indicated by their accession numbers. The DNA sequences retrieved in this work are indicated by their isolate code and accession number.

Fig. 3. DGGE analysis of yeast communities characterizing the kombucha fermented beverage stored at room temperature and at 4 °C for a period of 90 days. The numbers indicate sequenced DNA fragments and the colored circles their species affiliation. Marker (M).

Fig. 4. Cluster analysis of yeast DGGE profiles. Dendrograms obtained by UPGMA (Unweighted Pair Group Method Using Arithmetic Average) analysis, using Pearson's coefficient, based on yeast DGGE profiles obtained from the kombucha fermented beverage stored at room temperature (orange squares) and at 4 °C (blue squares) for a period of 90 days. Cophenetic correlation is shown at each node by numbers and coloured dots, ranging between green-yellow-orange-red, according to decreasing values. Standard deviation is shown at each node by a grey bar.

Fig. 5. Affiliation of the sequences retrieved from DGGE gel fragments (marked in Fig. 3) with the existing sequences of the partial D1/D2 region of the large sub-unit rRNA gene. Phylogenetic analysis was inferred by using the Maximum Likelihood method based on Jukes-Cantor model. Bootstrap (1000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA11. The sequences from the database are indicated by their accession numbers. The DNA

sequences retrieved in this work are indicated by their corresponding band number and their accession number. Symbols indicate samples analysed at time 0 (yellow square) and after 3 (circles) 20 (triangles) 45 (inverted triangles) and 90 days (diamonds) of storage at room temperature (orange) and at 4 °C (blue).

Fig. 6. DGGE analysis of AAB communities characterizing the kombucha fermented beverage stored at room temperature and at 4 °C for a period of 90 days. The numbers indicate sequenced DNA fragments and the colored circles their species affiliation. Marker (M).

Fig. 7. Cluster analysis of Acetic Acid Bacteria (AAB) DGGE profiles. Dendrograms obtained by UPGMA (Unweighted Pair Group Method Using Arithmetic Average) analysis, using Pearson's coefficient, based on AAB DGGE profiles obtained from the kombucha fermented beverage stored at room temperature (orange squares) and at 4 °C (blue squares) for a period of 90 days. Cophenetic correlation is shown at each node by numbers and coloured dots, ranging between green-yellow-orange-red, according to decreasing values. Standard deviation is shown at each node by a grey bar.

Fig. 8. Affiliation of the sequences retrieved from DGGE gel fragments (marked in Fig. 6) with the existing sequences of the with the existing 16S rRNA gene sequences. Phylogenetic analysis was inferred by using the Maximum Likelihood method based on the kimura 2-parameter model. Bootstrap (1000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA11. The sequences from the database are indicated by their accession numbers. The DNA sequences retrieved in this work are indicated by their corresponding band number and their accession number. Symbols indicate samples analysed at time 0 (yellow square) and after 3 (circles) 20 (triangles) 45 (inverted triangles) and 90 days (diamonds) of storage at room temperature (orange) and at 4 °C (blue).

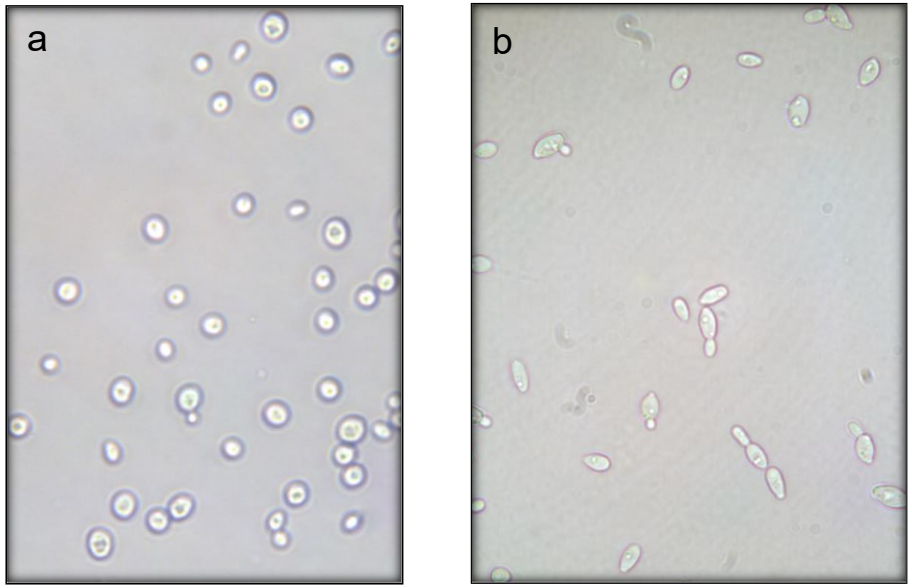
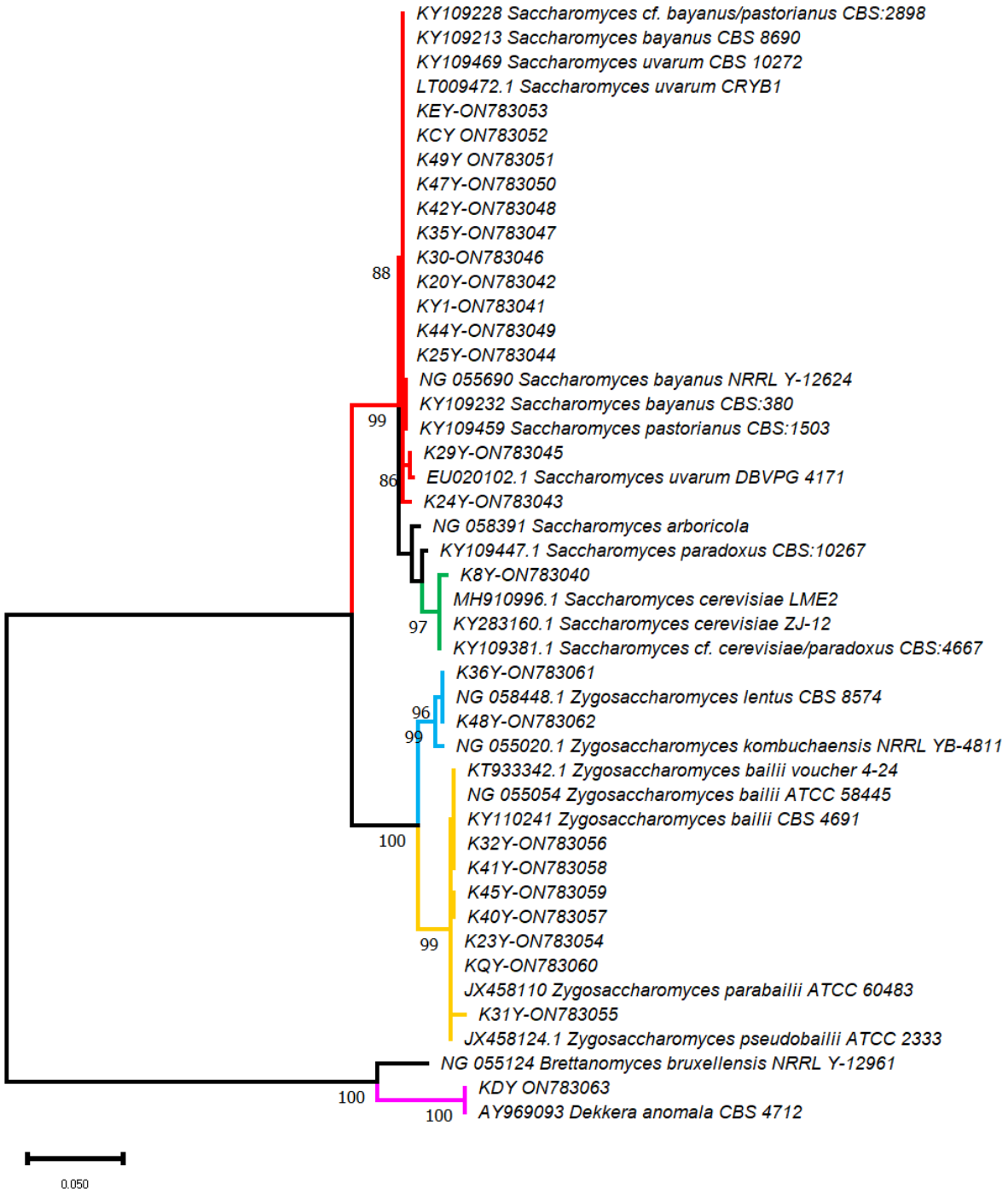


Fig. 1


KY109228 Saccharomyces cf. bayanus/pastorianus CBS:2898
KY109213 Saccharomyces bayanus CBS 8690
KY109469 Saccharomyces uvarum CBS 10272
LT009472.1 Saccharomyces uvarum CRYB1
KEY-ON783053
KCY ON783052
K49Y ON783051
K47Y-ON783050
K42Y-ON783048
K35Y-ON783047
K30-ON783046
K20Y-ON783042
KY1-ON783041
K44Y-ON783049
K25Y-ON783044
NG 055690 Saccharomyces bayanus NRRL Y-12624
KY109232 Saccharomyces bayanus CBS:380
KY109459 Saccharomyces pastorianus CBS:1503
K29Y-ON783045
EU020102.1 Saccharomyces uvarum DBVPG 4171
K24Y-ON783043
NG 058391 Saccharomyces arboricola
KY109447.1 Saccharomyces paradoxus CBS:10267
K8Y-ON783040
MH910996.1 Saccharomyces cerevisiae LME2
KY283160.1 Saccharomyces cerevisiae ZJ-12
KY109381.1 Saccharomyces cf. cerevisiae/paradoxus CBS:4667
K36Y-ON783061
NG 058448.1 Zygosaccharomyces lentus CBS 8574
K48Y-ON783062
NG 055020.1 Zygosaccharomyces kombuchaensis NRRL YB-4811
KT933342.1 Zygosaccharomyces bailii voucher 4-24
NG 055054 Zygosaccharomyces bailii ATCC 58445
KY110241 Zygosaccharomyces bailii CBS 4691
K32Y-ON783056
K41Y-ON783058
K45Y-ON783059
K40Y-ON783057
K23Y-ON783054
KQY-ON783060
JX458110 Zygosaccharomyces parabailii ATCC 60483
K31Y-ON783055
JX458124.1 Zygosaccharomyces pseudobailii ATCC 2333
NG 055124 Brettanomyces bruxellensis NRRL Y-12961
KDY ON783063
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97
96
99
100
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100
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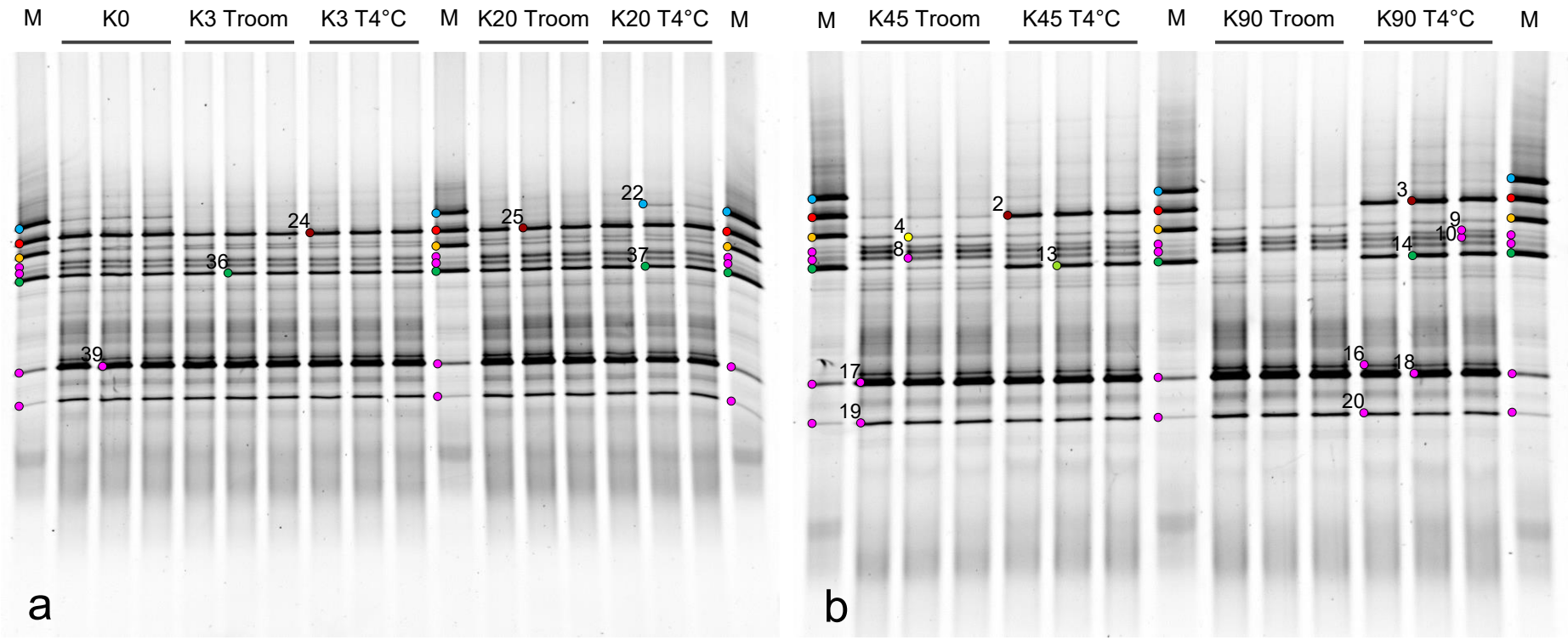
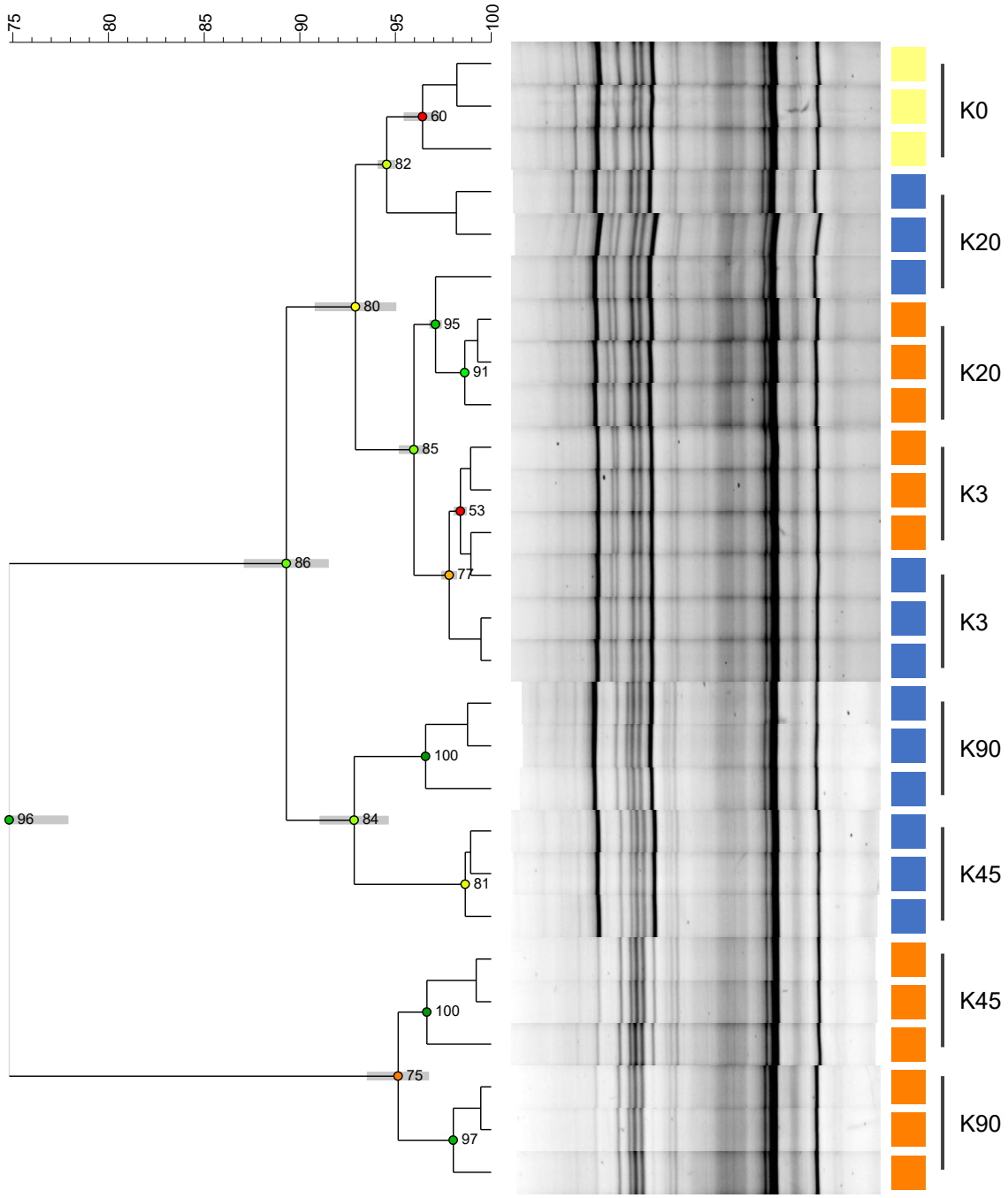
Fig. 2



- *D. anomala*
- *S. cerevisiae*
- *S. paradoxus/S. cerevisiae*
- *Z. bailii*
- *Z. bailii/Z. parabailii/Z. pseudobailii*
- *Z. lentus*
- *S. uvarum*
- *S. uvarum/S. eubayanus/S. pastorianus/S.eubayanus* x *S. uvarum/S. bayanus*

Fig. 3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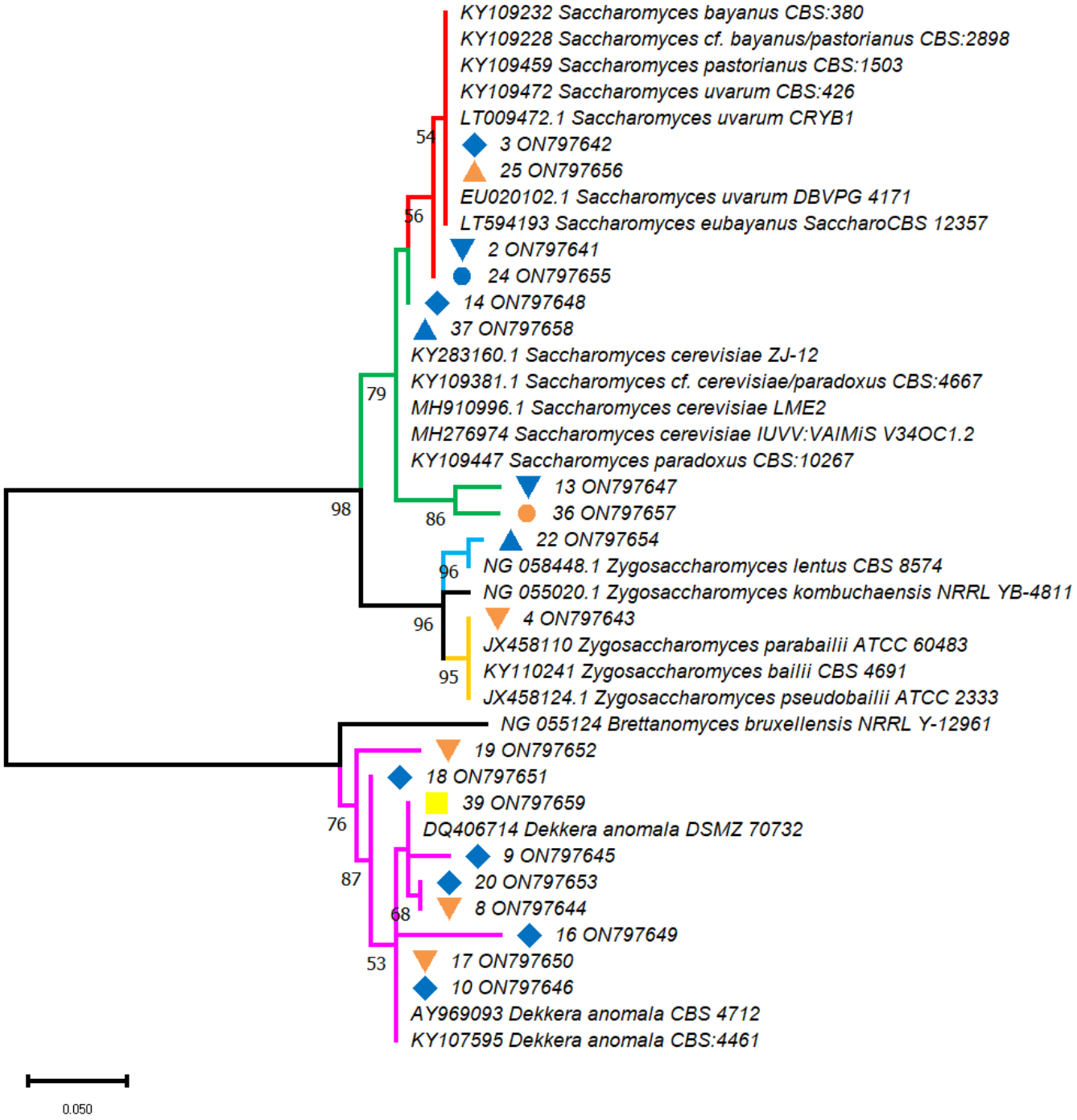
K20
K3
K3
K90
K45
K45
K90

Fig. 4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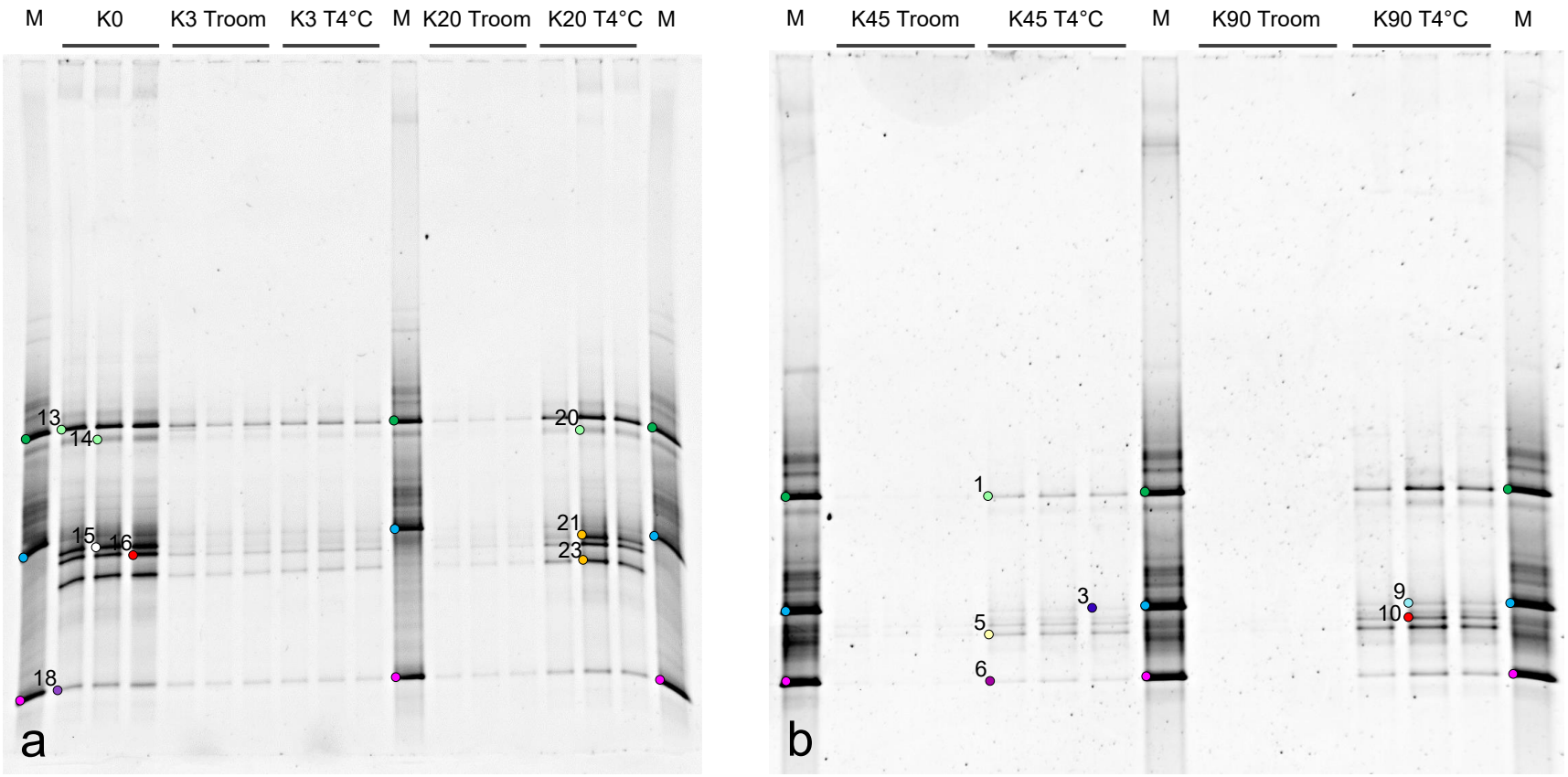
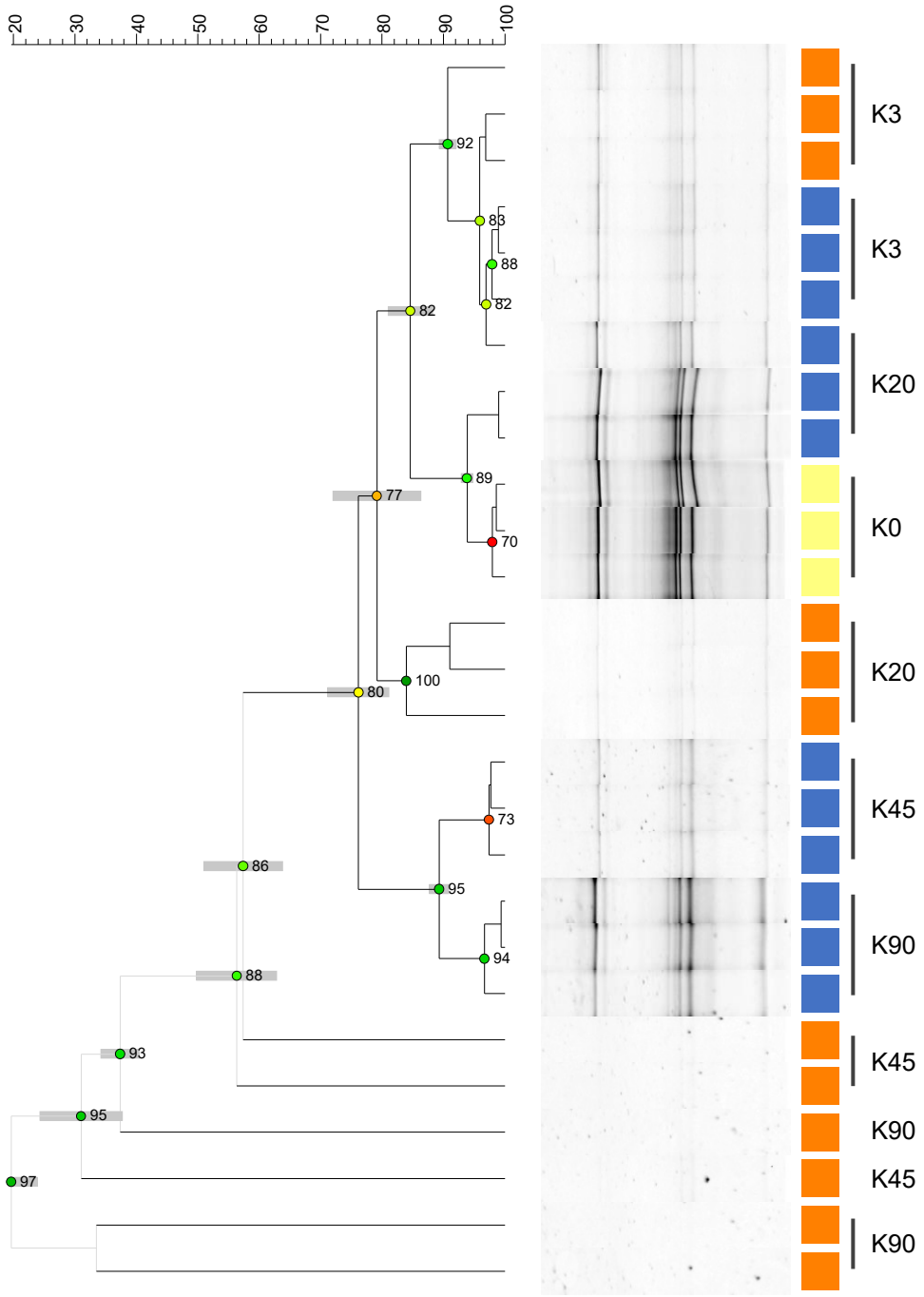
KY109228 Saccharomyces cf. bayanus/pastorianus CBS:2898
KY109459 Saccharomyces pastorianus CBS:1503
KY109472 Saccharomyces uvarum CBS:426
LT009472.1 Saccharomyces uvarum CRYB1
3 ON797642
25 ON797656
EU020102.1 Saccharomyces uvarum DBVPG 4171
LT594193 Saccharomyces eubayanus SaccharoCBS 12357
2 ON797641
24 ON797655
14 ON797648
37 ON797658
KY283160.1 Saccharomyces cerevisiae ZJ-12
KY109381.1 Saccharomyces cf. cerevisiae/paradoxus CBS:4667
MH910996.1 Saccharomyces cerevisiae LME2
MH276974 Saccharomyces cerevisiae IUVV:VAIMiS V34OC1.2
KY109447 Saccharomyces paradoxus CBS:10267
13 ON797647
36 ON797657
22 ON797654
NG 058448.1 Zygosaccharomyces lentus CBS 8574
NG 055020.1 Zygosaccharomyces kombuchaensis NRRL YB-4811
4 ON797643
JX458110 Zygosaccharomyces parabailii ATCC 60483
KY110241 Zygosaccharomyces bailii CBS 4691
JX458124.1 Zygosaccharomyces pseudobailii ATCC 2333
NG 055124 Brettanomyces bruxellensis NRRL Y-12961
19 ON797652
18 ON797651
39 ON797659
DQ406714 Dekkera anomala DSMZ 70732
9 ON797645
20 ON797653
8 ON797644
16 ON797649
17 ON797650
10 ON797646
AY969093 Dekkera anomala CBS 4712
KY107595 Dekkera anomala CBS:4461
54
56
79
98
86
96
96
95
76
87
68
53
0.050

Fig. 5



- *G. oxydans*
- *G. oxydans/ G. cerevisiae/G. aidae*
- *K. oboediens/k. rhaeticus/K. melomenusus*
- *K. oboediens/k. rhaeticus*
- *K. medellinensis/K. intermedius*
- *K. saccharivorans*
- *K. saccharivorans/K. xylinus*
- *K. saccharivorans/K. xylinus/K. diospyri*
- *K. mlaceti/K. europaeus*
- *N. hansenii*
- *N. hansenii/Ga. entanii*

Fig. 6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100
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73
86
95
94
88
93
95
97
K3
K3
K20
K0
K20
K45
K90
K45
K90
K45
K90

Fig. 7

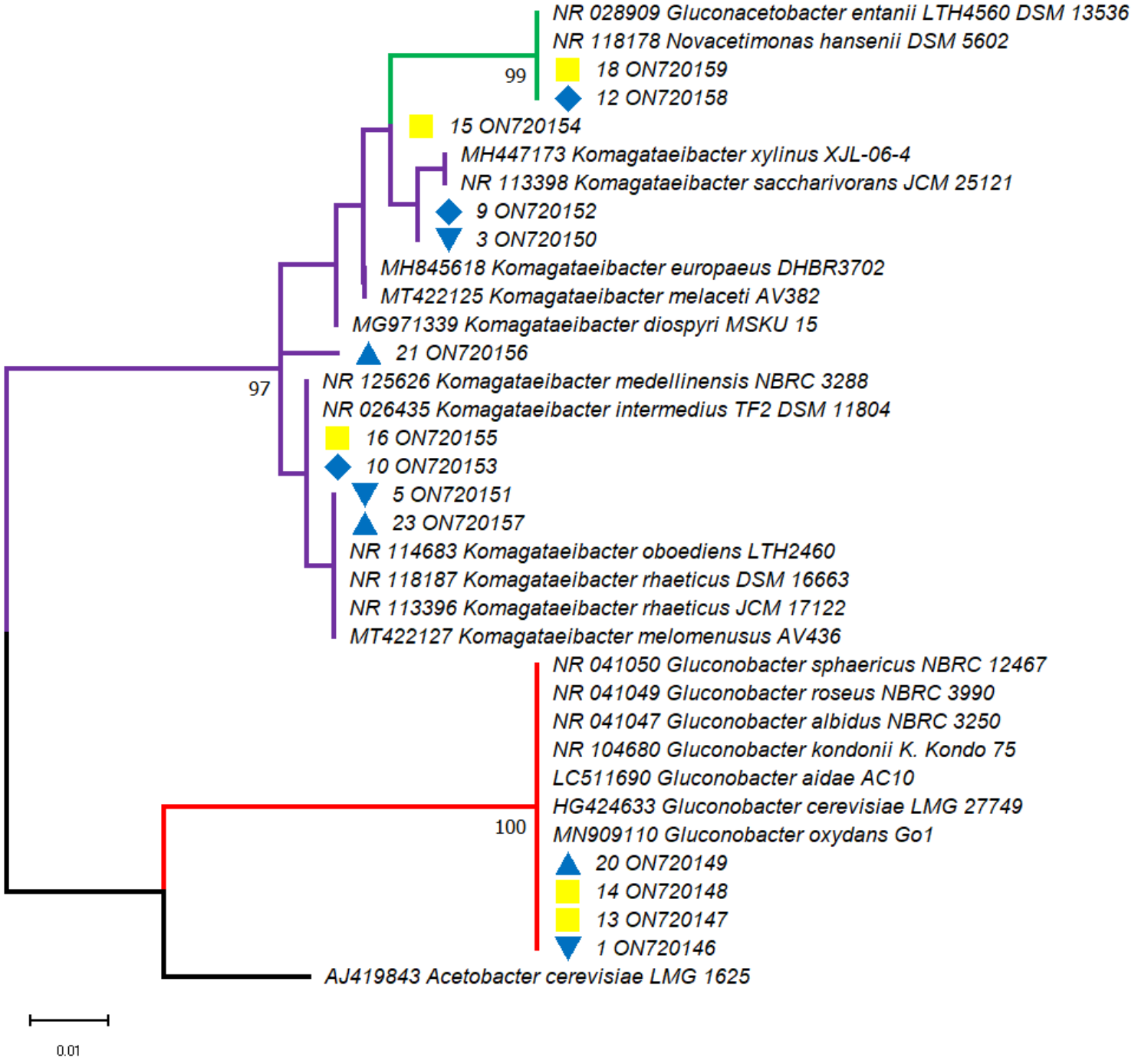
NR 028909 Gluconacetobacter entanii LTH4560 DSM 13536
NR 118178 Novacetimonas hansenii DSM 5602
99
18 ON720159
12 ON720158
15 ON720154
MH447173 Komagataeibacter xylinus XJL-06-4
NR 113398 Komagataeibacter saccharivorans JCM 25121
9 ON720152
3 ON720150
MH845618 Komagataeibacter europaeus DHBR3702
MT422125 Komagataeibacter melaceti AV382
MG971339 Komagataeibacter diospyri MSKU 15
21 ON720156
97
NR 125626 Komagataeibacter medellinensis NBRC 3288
NR 026435 Komagataeibacter intermedius TF2 DSM 11804
16 ON720155
10 ON720153
5 ON720151
23 ON720157
NR 114683 Komagataeibacter oboediens LTH2460
NR 118187 Komagataeibacter rhaeticus DSM 16663
NR 113396 Komagataeibacter rhaeticus JCM 17122
MT422127 Komagataeibacter melomenusus AV436
NR 041050 Gluconobacter sphaericus NBRC 12467
NR 041049 Gluconobacter roseus NBRC 3990
NR 041047 Gluconobacter albidus NBRC 3250
NR 104680 Gluconobacter kondonii K. Kondo 75
LC511690 Gluconobacter aidae AC10
HG424633 Gluconobacter cerevisiae LMG 27749
100
MN909110 Gluconobacter oxydans Go1
20 ON720149
14 ON720148
13 ON720147
1 ON720146
AJ419843 Acetobacter cerevisiae LMG 1625
0.01

Fig. 8

Storage time and temperature affect microbial dynamics of yeasts and acetic acid bacteria in a kombucha beverage

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Fig. S1. Microbiological analyses of the kombucha beverage during 90 days of storage at room temperature (orange) and at 4 °C (blue). Fast growing yeasts (*Saccharomyces* spp.) on WL agar medium **(a)** and on SDA medium **(b)**; Slow growing yeasts (*Zygosaccharomyces* spp. and *D. anomala*) on WL agar medium **(c)** and on SDA medium **(d)**; AAB on WL agar medium **(e)** and on YPM medium **(f)**; LAB on MRS medium **(g)**; Total mesophilic aerobic counts on PCA medium **(h)** (***, p ⩽ 0 0.001; **, p ⩽ 0.01; *, p ⩽ 0.05).

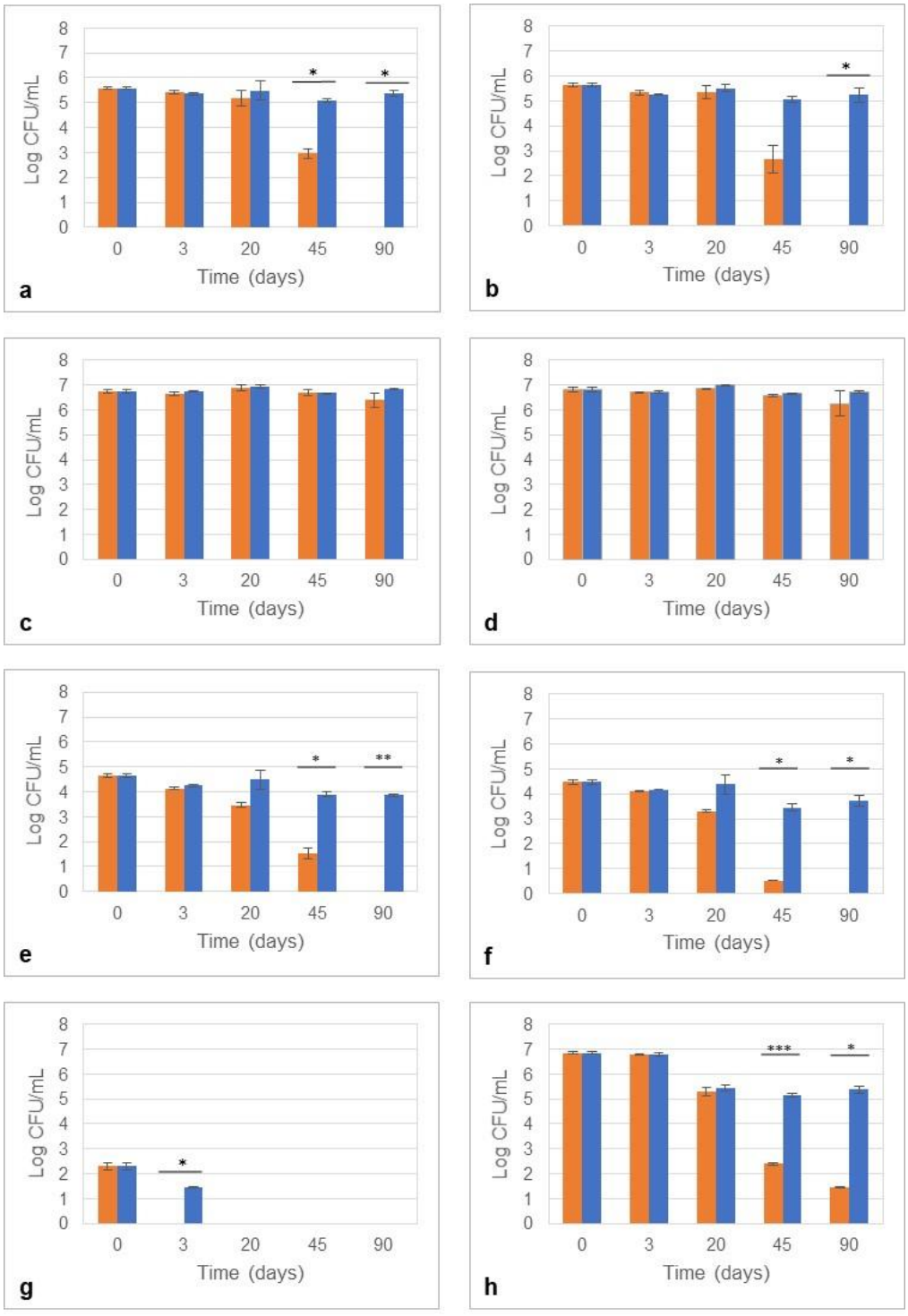

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Fig. S2. Species-specific PCR for *D. anomala* of the 39 slow-growing yeast isolated from kombucha, using pA1/ITS4 primers. 1: *D. anomala* ATCC 10562; 2: IMA K31Y; 3: IMA KQY; 4: K22Y; 5: K48Y; 6-11: IMA K2Y-K7Y; 12-19: IMA K10Y-K17Y; 20-21: IMA K40Y-K41Y; 22: IMA K45Y; 23: IMA KFY; 24-26: IMA K18Y-K21Y; 27: IMA K23Y; 28: IMA K27Y; 29-31: IMA K32Y-K34Y; 32-35: IMA K36Y-K39Y; 36: IMA KDY; 37-39: IMA KNY-KPY; 40: IMA KHY; C: negative control; M: Marker 100 bp (Thermo Scientific™).

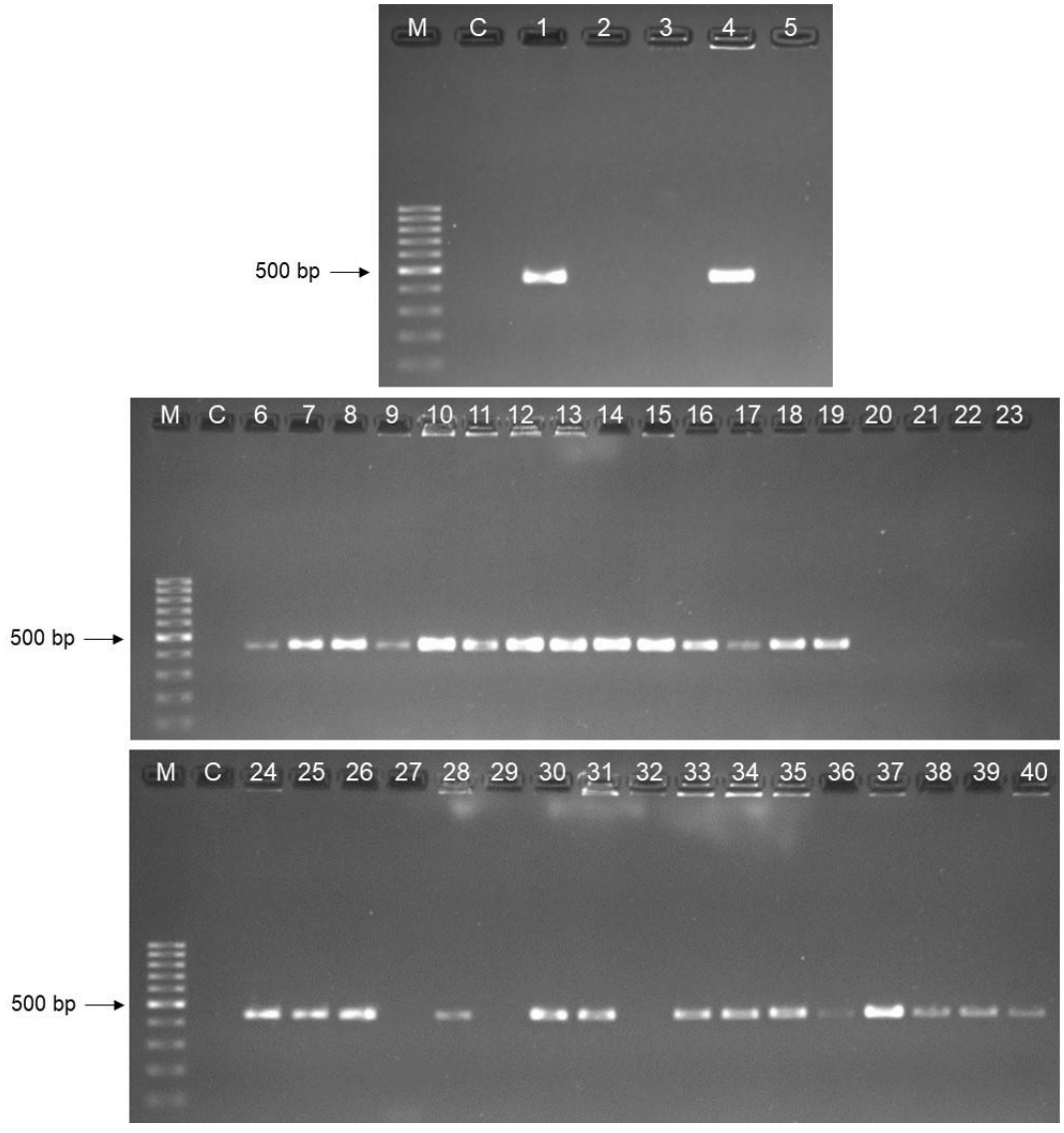

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Fig. S3. ITS amplification products of fast-growing yeast (*Saccharomyces* spp.) along with the *D. anomala* IMA KDY, isolated from kombucha, using ITS1/ITS4 primers. 1: IMA K1Y; 2: IMA K8Y; 3: IMA K20Y; 4-7: IMA K23Y-K26Y; 8-12: IMA K28Y-K32Y; 13-14: IMA K35Y-K36Y; 15-24: IMA K40Y-K49Y; 25-27: IMA KCY- KEY; 28-29: IMA KQY-KRY; C: negative control; M: Marker 100 bp (BioLabs).

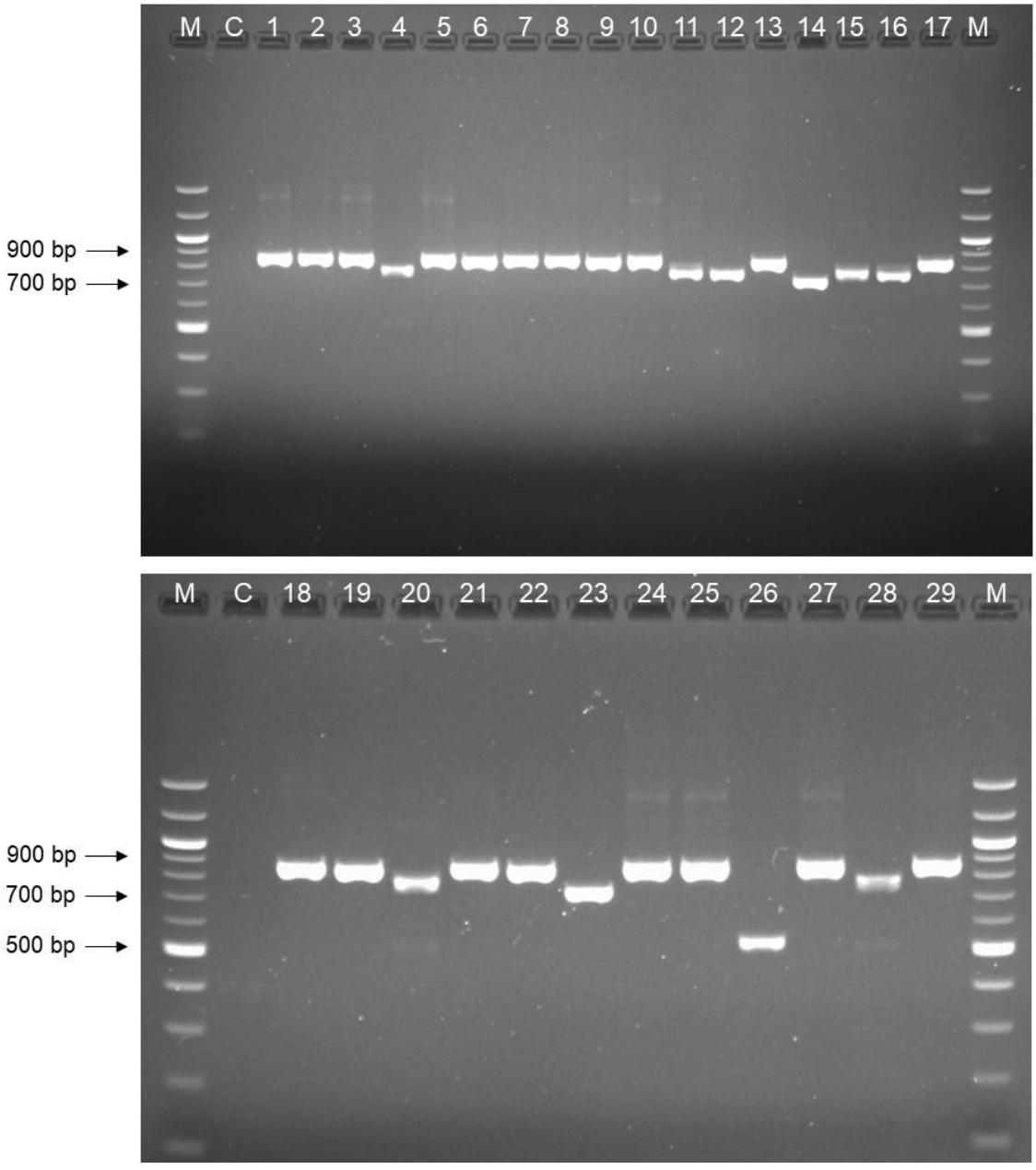

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Fig. S4. *Hae*III and *Hpa*II RFLP of the ITS regions of *Saccharomyces* strains isolated from kombucha. 1: IMA K1Y; 2: IMA K8Y; 3: IMA K20Y; 4-6: IMA K24Y-K26Y; 7-9: IMA K28Y-K30Y; 10: IMA K35Y; 11-13: IMA K42Y-K44Y; 14-15: IMA K46Y- K47Y; 16: IMA K49Y; 17: IMA KCY; 18: IMA KEY; 19: IMA KRY; M: Marker 100 bp (BioLabs).