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Effects of intrinsic microbial stress factors on viability and physiological condition of yeasts isolated from spontaneously fermented cereal doughs

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ABSTRACT

Fermented cereal doughs constitute a predominant part of West African diets. The environment of fermented doughs can be hostile for microbial survival due to high levels of microbial metabolites such as weak carboxylic organic acids and ethanol. In order to get a better understanding of the intrinsic factors affecting the microbial successions of yeasts during dough fermentation, survival and physiological responses of the yeasts associated with West African fermented cereal doughs were investigated at exposure to relevant concentrations of microbial inhibitory compounds. Three strains each of the predominant species, i.e. *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Pichia kudriavzevii* as well as the opportunistic pathogen *Candida glabrata* were studied. The strains were exposed to individual stress factors of cereal doughs, i.e. (i) pH 3.4, (ii) 3% (*v*/v) ethanol ($EtOH_{pH3.4}$), (iii) 285 mM lactic acid ($LA_{pH3.4}$) and (iv) 150 mM acetic acid ($AA_{pH3.4}$) as well as to combinations of these stress factors, i.e. (v) $(LA + AA)_{pH\,3.4}$ and (vi) $(LA + AA + EtOH)_{pH\,3.4}$. Growth and single cell viability were studied by flow cytometry using combined SYTO 13 and propidium iodide (PI) staining. Intracellular pH (pH_i), plasma membrane integrity and micro-colony development of stressed cells were studied by fluorescence microscopy using PI and carboxyfluorescein diacetate succinimidyl ester (CFDA-se).

Viability of the yeast strains was not affected by pH 3.4 and 3% (*v*/v) ethanol ($EtOH_{pH3.4}$). 285 mM lactic acid ($LA_{pH3.4}$) reduced the specific growth rate (μ_{max}) from 0.27-0.41 h^{-1} to 0.11–0.26 h^{-1} and the viability from 100% to 2.6–41.7% at 72 h of exposure in most yeast strains, except for two strains of *C. glabrata*. 150 mM acetic acid ($AA_{pH3.4}$) as well as the combinations $(LA + AA)_{pH\,3.4}$ and $(LA + AA + EtOH)_{pH\,3.4}$ reduced μ_{max} to 0.0 h^{-1} and induced significant cell death for all the yeast strains. Exposed to $(LA + AA + EtOH)_{pH\,3.4}$, the most resistant yeast strains belonged to *S. cerevisiae* followed by *P. kudriavzevii*, whereas *C. glabrata* and *K. marxianus* were more sensitive. Strain variations were observed within all four species. When transferred to non-stress conditions, i.e. MYGP, pH 5.6, after exposure to $(LA + AA + EtOH)_{pH\,3.4}$ for 6 h, 45% of the single cells of the most resistant *S. cerevisiae* strain kept their plasma membrane integrity, recovered their pH_i to near physiological range (pH_i = 6.1–7.4) and resumed proliferation after 3–24 h of lag phase. The results obtained are valuable in order to change processing conditions of the dough to favor the survival of preferable yeast species, i.e. *S. cerevisiae* and *K. marxianus* and inhibit opportunistic pathogen yeast species as *C. glabrata*.

1. Introduction

Cereal grains mainly maize, sorghum, millet and rice nourish millions of people in the West African region and constitute the most important staple food in this part of the world (Nout, 2009). The grains are generally soaked, milled and processed at room temperature (27–33 °C) by spontaneous fermentation into doughs at household level or at small industrial scale. The doughs (e.g., ogi, mawè and similar products) serve for the preparation of indigenous fermented foods and/or beverages that are important parts of the nutrition of West African people (Houngbédji et al., 2019; Hounhouigan et al., 1993; Jespersen et al., 1994). During cereal dough fermentations lactic acid bacteria produce weak carboxylic acids that acidify the dough to a pH generally below 4.0 while yeasts produce ethanol (Hounhouigan et al., 1993; Martynova et al., 2016; Nout, 2009). Lowering of pH and accumulation of weak carboxylic acids and ethanol make the microenvironment of the dough hostile for microbial survival. Lactic acid and acetic acid have been reported as the most significant weak carboxylic acids in

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cereal doughs. The concentration of lactic acid in fermented maize dough has been reported as 14 g/kg (Halm et al., 1993) which at a pH of 3.7 and a dry matter content of 50%, according to the Henderson-Hasselbalch Equation, corresponds to a level of 187 mM undissociated lactic acid (316 mM total lactic acid) in the water phase (Halm et al., 2004). Michodjèhoun-Mestres et al. (2005) reported the concentrations of total lactic acid (23.9 mg/g corresponding to 265.3 mM) and total acetic acid (2.0 mg/g corresponding to 33.3 mM) in fermented sorghum dough at pH 3.7.

Previous studies have identified several yeast species being associated with West African spontaneously fermented cereal doughs (Greppi et al., 2013a, 2013b; Hayford and Jespersen, 1999; Houngbédji et al., 2018; Hounhouigan et al., 1994). *Pichia kudriavzevii* was found as the most abundant yeast species (56–92% of total yeast species), followed by *Kluyveromyces marxianus* (11–28% of total yeast species) and *Saccharomyces cerevisiae* (6–22% of total yeast species) during spontaneous fermentation of mawè, a cereal dough very popular in West Africa (Greppi et al., 2013a, 2013b; Houngbédji et al., 2018; Hounhouigan et al., 1994). These yeast species are preferable as they display desirable technological, nutritional, sensory and even probiotic properties during the fermentation (Jespersen, 2003, 2005; Pedersen et al., 2012; Greppi et al., 2016). As yeast are generally resistant to acidic stress (Sugiyama et al., 2015), the high acid tolerance of the above desirable yeasts constitutes an advantage for cereal fermentations. However, the resistance of yeasts to acidic stress conditions poses a great challenge for food preservation when it comes to spoilage yeasts and opportunistic pathogenic yeast species as e.g. *Candida glabrata* which occasionally has been associated with fermented cereal doughs (Houngbédji et al., 2018; Hounhouigan et al., 1993).

The viability and stress responses of yeasts to the fermented cereal dough conditions have been sparsely studied. Nevertheless, numerous studies have been devoted to yeast viability and stress response mechanisms towards lactic acid stress and/or acetic acid stress using *S. cerevisiae* as yeast model (Giannattasio et al., 2013; Halm et al., 2004; Mira et al., 2010; Narendranath et al., 2001b). Previously, we investigated tolerances of single cells of *P. kudriavzevii* (formerly named *Issatchenkia orientalis*, anamorph *C. krusei*) and *S. cerevisiae* isolated from fermented maize dough to lactic acid concentrations occurring in fermented maize doughs (Halm et al., 2004). However, only few studies have focused on other non-*Saccharomyces* yeast species associated with cereal dough fermentations as e.g. *K. marxianus* (Martynova et al., 2016) as well as associated yeast contaminants as e.g. *C. glabrata* (Ullah et al., 2013a). Given that the food quality and safety highly depend on the diversity and viability of microorganisms that occur, it is of great interest to investigate the survival of all these yeast species when exposed to the combination of stress factors of cereal doughs.

Upon exposure to weak organic acids at pH below the dissociation constant (pKa = 3.86 for lactic acid and 4.74 for acetic acid), the undissociated molecules of the weak acids (RCOOH) predominates and permeates the cell where it dissociates into acid anions ($RCOO^-$) and protons (H^+) inducing acidification of the cytosol (Giannattasio et al., 2013; Halm et al., 2004). To counteract cytosolic acidification, the yeast cell upregulate resistance mechanisms for pumping out the protons at the cost of ATP, inducing significant loss of available energy for growth and other essential metabolic functions (Giannattasio et al., 2013). Previously, we reported that sensitivity and resistance mechanisms could differ from species to species, from strain to strain, and even cells within a strain could react differently towards stress conditions (Arneborg et al., 2000; Halm et al., 2004; Zhao et al., 2017). Elucidation of the viability and stress resistance responses of yeasts towards conditions mimicking those encountered in cereal doughs can influence strategies for improving quality and safety of the doughs. Furthermore, deeper knowledge on yeast survival in such stressful environments can help finding ways to optimize fermentation processing to change the environment of fermented cereal doughs in favor of the preferred yeast species.

The overall aim of this study was to get an in depth understanding of how various stress factors in mawè, a West African spontaneous fermented cereal-based dough, influence the growth and survival of the predominant yeast species and to explore differences in sensitivity at species and strain level. The knowledge obtained will enable the control of the spontaneous fermentation in a manner so that the preferred yeast species will have better proliferation while growth of opportunistic pathogenic yeasts will be inhibited during the fermentation.

2. Materials and methods

2.1. Production and characterization of two types of mawè doughs

2.1.1. Processing and fermentation conditions

Two types of commercial mawè doughs based on maize and sorghum respectively were processed by the local producers using processing methods described earlier (Houngbédji et al., 2018) with slight modification as reported in Fig. S1. In summary, the maize or sorghum grains were sorted, washed and dehulled into grits. The grits was sorked for 6–14 h depending on producers, milled, kneaded and water content adjusted to approx. 50%. The resulting fresh mawè was fermented spontaneously in plastic containers at room temperature (28–32 °C). For each mawè type, the dough was sampled from two production sites each in duplicate at the onset (0 h), at 6 h, 12 h, 24 h, 48 h and at the end of the fermentation (72 h). The samples were aseptically transported on ice from the production sites to the laboratory and directly analyzed.

2.1.2. Determination of pH, weak organic acid content and ethanol content of mawè doughs

Determination of pH was carried out by homogenizing 10 g of mawè dough in 20 mL of distilled water, followed by pH measurement using an InoLab digital pH-meter (3505 pH meter, JENWAY). Weak organic acid content and ethanol content were determined by HPLC, as described by Michodjèhoun-Mestres et al. (2005) with slight modification. In summary, the HPLC system (Knauer system, Germany) was equipped with a Supelcogel H 59304-U Column (30 cm × 7.8 mm ID, Bellefonte, PA, USA) with Supelguard C610H pre-column (5 cm × 4.6 mm ID). The HPLC system was standardized with standard solutions at 1 and 10 mg/mL of organic acids (acetate and lactate) and ethanol. Elution was performed at 60 °C with diluted sulphuric acid (5 mM) at 0.6 mL/min. For determination of the organic acids (lactic and acetic acids) and ethanol concentrations during fermentations of mawè doughs, 400 mg of wet mawè (51% moisture content) were vigorously suspended in 1 mL of 5 mM H_2SO_4 and stirred at ambient temperature (28–30 °C) for 30 min. The suspension was then centrifuged at 7000 ×*g* for 10 min. The supernatant was filtered through 0.45 μm pore size filters and 100 μL was then injected onto the HPLC system. Organic acid detection was performed by UV absorption at 210 nm with a Spectra system UV2000 and alcohol detection by refractometry with Smartline RI Detector 2300 (n°110,542, Knauer, Germany).

2.2. Yeast strains and growth media

Three strains each of *Saccharomyces cerevisiae* (Sc1, Sc2, and Sc3), *Kluyveromyces marxianus* (Km1, Km2 and Km3), *Pichia kudriavzevii* (Pk1, Pk2 and Pk3) and *Candida glabrata* (Cg1, Cg2 and Cg3) were used in this study. Further details on the twelve strains are summarized in Table 1. The strains were previously isolated and identified during spontaneous fermentation of mawè doughs (Houngbédji et al., 2018) and were maintained in 20% (*v*/v) glycerol at −80 °C.

MYGP medium (3 g/L of yeast extract (Difco), 3 g/L of malt extract (Difco), 5 g/L of bactopeptone (Difco) and 10 g/L of glucose) with pH adjusted to 5.6 and 3.4 were used in the control (non-stress condition) and stress experiments, respectively. Stress experiments were performed using MYGP medium at six different conditions: (i) low pH

Table 1
Yeast strains included in the study.

Abbreviation	Identity	Isolate source[a]	Strain code[a]	NCBI GenBank accession no[a]
Sc1	*Saccharomyces cerevisiae*	Undehulled maize mawè, fermented for 36 h	Cm2-36-Y11	MG245859
Sc2	*Saccharomyces cerevisiae*	Commercial maize mawè, at onset of fermentation	C2-0-Y2	MG245839
Sc3	*Saccharomyces cerevisiae*	Undehulled maize mawè, fermented for 36 h	Cm1-36-Y6	MG245858
Km1	*Kluyveromyces marxianus*	Commercial maize mawè, at onset of fermentation	C1-0-Y6	MG245826
Km2	*Kluyveromyces marxianus*	Commercial sorghum mawè, fermented for 6 h	S1-6-Y4a	MG245824
Km3	*Kluyveromyces marxianus*	Homemade maize mawè, at onset of fermentation	H2-0-Y2	MG245846
Pk1	*Pichia kudriavzevii*	Homemade maize mawè, at onset of fermentation	H1-0-Y5	MG245834
Pk2	*Pichia kudriavzevii*	Commercial sorghum mawè, fermented for 6 h	S2-6-Y4	MG245830
Pk3	*Pichia kudriavzevii*	Homemade maize mawè, fermented for 12 h	H2-12-Y1	MG245831
Cg1	*Candida glabrata*	Commercial maize mawè, fermented for 6 h	C2-6-Y2	MG245841
Cg2	*Candida glabrata*	Commercial maize mawè, at onset of fermentation	C2-0-Y3	MH753705
Cg3	*Candida glabrata*	Commercial maize mawè, fermented for 24 h	C2-24-Y3	MG245821

[a] Houngbédji et al. (2018).

stress (pH 3.4); (ii) ethanol stress ($EtOH_{pH3.4}$), with 3% (*v*/v) ethanol (Plum, Assens, Denmark); (iii) lactic acid stress ($LA_{pH3.4}$), with 285 mM total lactic acid (Sigma); (iv) acetic acid stress ($AA_{pH3.4}$), with 150 mM total acetic acid (Sigma); (v) the combination of lactic acid stress and acetic acid stress ($(LA + AA)_{pH\ 3.4}$), with a mixture of 285 mM LA and 150 mM AA and (vi) the combination of lactic acid stress, acetic acid stress and ethanol stress ($(LA + AA + EtOH)_{pH\ 3.4}$), with a mixture of 285 mM LA, 150 mM AA and EtOH 3% (*v*/v).

2.3. Preparation of yeast cell suspensions

Yeast strains were spread plated on MYGP agar, pH 5.6 and incubated aerobically at 25 °C for 48 h. A single colony was cultivated in 10 mL MYGP broth, pH 5.6 at 25 °C for 24 h. 500 μL of the 24 h culture was propagated in 50 mL MYGP broth, pH 5.6 incubated overnight (16 h) at 25 °C. Cell concentration of the overnight culture was calculated by bright field microscopy using a Neubauer counting chamber. A volume containing 5×10^7 cells/mL of the overnight culture was taken out and cells were harvested by centrifugation (12,000 ×*g*, 5 min) (154RF, Ole Dich, Denmark). The harvested cells were washed once in saline peptone [1% NaCl, *w*/w; (Merck) and 5 g/L of bactopeptone], and re-suspended in 10 mL of each of the seven media, i.e. the six stress conditions and the non-stress condition, resulting in a final concentration of 5×10^6 cells/mL; OD_{600} 0.2–0.3 for each of the cell suspensions. The resulting cell suspensions were used simultaneously in growth performance, flow cytometry and plate counting experiments as illustrated in Fig. S2.

2.4. Determination of growth performance

200 μL from of each of the cell suspensions was transferred to 96-wells microplates (Corning ®, Sigma-Aldrich, Schnelldorf, Germany) in triplicate and growth curves were assessed from two biological replicates at 25 °C for 24 h by measuring OD_{600} every hour using a Varioskan™ Flash (Thermo Fisher Scientific, Oy, Finland). The OD_{600} values were ln-transformed after multiplying by a constant factor (100) and growth curves were established using the $\ln(100{*}OD_{600})$ values. The maximum specific growth rate (μ_{max}, h^{-1}) was determined by fitting the growth curves using DMfit software (Institute of Food Research, Norwich, UK).

2.5. Staining and flow cytometric analysis

The remain of the cell suspensions (9.4 mL for each with 5×10^6 cells/mL) for the non-stress condition and the six stress conditions prepared as described above was incubated at 25 °C and the cell suspensions were shaked and sampled at four different incubation time points, i.e. at 6 h, 24 h, 48 h and 72 h and analyzed for cell viability. Cells were stained 30 min before the end of each incubation time point by sampling 1 mL of the cell suspensions and simultaneously adding 2 μL green SYTO 13 5.0 mM (Molecular Probes S7575, Invitrogen, stock 5 mM in DMSO) and 10 μL red Propidium Iodide (PI) 1.5 mM (Molecular Probes P3566, Invitrogen, stock 1.0 mg/mL in water) followed by incubation at 30 °C for 30 min in the dark. The dual-stained cell suspensions were diluted 10 times with 1% (*w*/*v*) saline peptone, pH 5.6 and kept on ice until analysis.

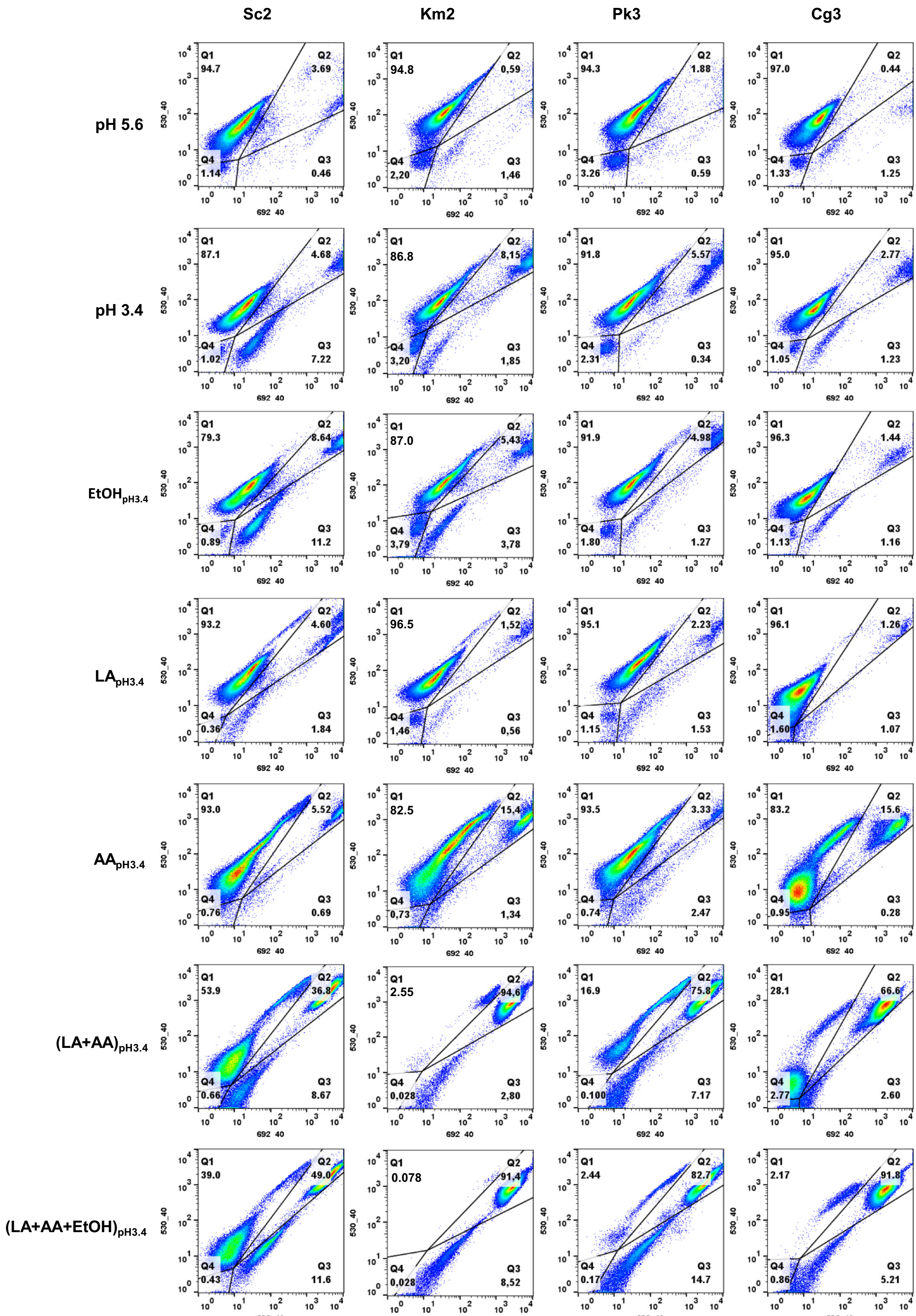
Flow cytometric analyses were performed as previously described by Zhao et al. (2017). In brief, samples were analyzed by a BD FACS Jazz Cell Sorter (BD Biosciences, USA) with a 488 nm argon ion laser. Green fluorescence emitted from SYTO 13 stained cells was collected with bandpass filter 530_40 nm, whereas the red fluorescence emitted form PI stained cells was collected with bandpass filter 692_40 nm. Events were collected by triggering on the side scatter channel analyzing one mL of dual-stained cell suspension recording 100,000 events per sample. Data were collected and stored in BD FACS Software sorter software (BD Biosciences, USA). The collected data were analyzed with the FlowJo_V10 (Tree Star, Inc. USA). Four quadrants were defined clockwise starting from the top-left one as Q1, Q2, Q3, and Q4 on the dot plot images, as indicated on Fig. 1.

2.6. Determination of CFU counts and calculation of log addition and log reduction

Simultaneously with flow cytometric analysis, 500 μL of each of the seven cell suspensions was sampled at the incubation time points (0 h, 6 h, 24 h, 48 h and 72 h), serially diluted in 4.5 mL 1% (w/v) saline peptone, pH 5.6 and 100 μL spread plated on MYGP agar, pH 5.6, incubated at 25 °C for 48–72 h. Growth and log reduction was defined as the difference between the log CFU counts at 0 h and at the incubation time point (6 h, 24 h, 48 h and 72 h) for each cell suspension.

2.7. Fluorescence staining for pH_i measurements

A volume containing 5×10^7 cells of the overnight culture (16 h) prepared as described above, was harvested by centrifugation (12,000 ×*g*, 5 min). Cells were washed once in 1% (w/v) saline peptone, pH 5.6, harvested and re-suspended in 980 μL citrate phosphate buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid), pH 5.6 added 10 μL 1 M glucose (Merck). Cells were stained by adding 10 μL carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) 4.4 mM (Life Technologies C1157, stock 25 mM in DMSO), incubated in dark for 30 min at 25 °C and harvested by centrifugation (12,000 ×*g* for 5 min). Cells were re-suspended in 10 mL of stress medium $(EtOH + LA + AA)_{pH\ 3.4}$ mimicking the combination of stress factors of fermented mawè dough, incubated at 25 °C for 6 h. At the incubation time point, one mL of the stressed cell culture was harvested by centrifugation (12,000 ×*g*, 5 min) and the stressed cells were re-suspended in citrate phosphate buffer pH 5.6 containing 10% (*w*/*v*) glucose and analyzed after 30 min.



(caption on next page)

Fig. 1. Density plots of the most resistant strain (Sc2, Km2, Pk3 and Cg3) of each examined yeast species after 6 h exposure to the stress conditions, i.e. low pH (pH 3.4), 3% (*v*/*v*) ethanol ($EtOH_{pH3.4}$), 285 mM total lactic acid ($LA_{pH3.4}$), 150 mM total acetic acid ($AA_{pH3.4}$), the combination stress conditions $(LA + AA)_{pH\ 3.4}$ and $(LA + AA + EtOH)_{pH\ 3.4}$. The SYTO 13 signal was recorded in the green channel (530_40) on the Y-axis and the PI signal was recorded in the red channel (692_40) on the X-axis. Events in different quadrants correspond to different populations: viable cells (Q1), dead cells (Q2), intermediate population with damaged cell membranes (Q3) and weakly stained cells (Q4). The number in each quadrant represents the relative percentage of each population. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To construct the calibration curves for pH_i determination, 70% (*v*/v) ethanol were added to non-stressed CFDA-se stained cells for 30 min to achieve fully permeabilized cells. Subsequently, permeabilized cells were harvested by centrifugation (12,000 ×*g*, 5 min) and re-suspended in citrate phosphate buffer with pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The average ratio of at least 60 cells for each pH value was plotted against the $Ratio_{(490/435\ nm)}$, and the standard error of the mean was calculated. A relationship between pH_i and $R_{490/435}$ was set up for the range from pH 5.0 to 8.0. Three-order polynomial interpolation between the calibration points was used to calculate the pH_i of each cell and ratios < 0.68 for Sc2 and 0.72 for Km1 were recorded as pH < 5 (Fig. S3).

2.8. pH_i measurements, plasma membrane integrity and micro-colony formation

For stressed cell cultures pH_i measurements, plasma membrane integrity and micro-colony formation were measured in a CellASIC ONIX Y04C microfluidic yeast plate (height of 3.5 to 4.5 μm; Merck Millipore), primed with fresh MYGP pH 5.6, using 5 psi for 5 min. The plate was connected to an ONIX Microfluidic system (CellASIC EV262 system) and a microincubator controller (MIC230) maintaining 25 °C inside the plate during the experiment. The plate was placed in an inverted fluorescence microscope (Zeiss Axiovert 135) equipped with a 40× objective (numerical aperture, 1.3). The cells were excited with a Hg lamp at 490 and 435 nm using a dichroic mirror (510 nm), and an emission band pass filter (515 to 565 nm). The fluorescence emission was recorded with a ccd-camera (Evolution VF, Qimaging) with an exposure time of 500 ms, using the software Image Pro 5.0. 200 μL of the CFDA-se stained stressed cells were loaded into the chamber at 5 psi for 10 s and un-trapped cells were washed out at 5 psi for 5 min. The flow rate was adjusted to 1 psi from the start to the end of the experiment (24 h). The start of the experiment (0 min) was set upon flushing the cells with MYGP, pH 5.6. Fluorescence intensities of individual cells were recorded after 30 min and 90 min by capturing the fluorescent images and further analyzed. Additionally, bright-field images were captured to monitor the micro-colony formation of individual cell for 24 h of incubation.

2.9. Data analysis of pH_i measurements

Calculation of pH_i values were performed as previously described (Budde and Jakobsen, 2000; Siegumfeldt et al., 1999). The fluorescent images were analyzed with the image analysis software image J (version1.48; National Institutes of Health (NIH), Bethesda, MD, USA). Regions along the perimeters of the cells were drawn, and a ratio $R_{490/435}$ was determined by dividing the fluorescence intensity at 490 nm by the fluorescence intensity at 435 nm for each pixel of a cell image. The pH_i values were determined using the $R_{490/435}$ and a calibration curve for each strain (Fig. S3).

2.10. Data analysis and statistics

Data were collected in two or three replicates depending on the experiment and means and standard deviations were calculated. Means of organic acids and ethanol concentrations in mawè doughs fermented for 0 h, 6 h, 12 h, 24 h, 48 h and 72 h were separated using one-way analysis of variance (ANOVA) and least significant difference was determined with Fisher statistic ($P < 0.05$) provided by STATISTICA V7.1 software. For the maximum specific growth rate (μ_{max}, h^{-1}) and the viable proportion of yeast strains, means calculated for stress conditions were each compared to the reference i.e. non-stress condition using Student's test of conformity and significant differences were reported for α = 0.05, 0.01 and 0.001. The correlations between culturability (CFU) and viability at species and strain level were calculated using Pearson statistic provided by the MINITAB 18 statistical software package (MINITAB Inc. Release 17 for windows, 2015). The correlation curves between culturability and viability was constructed by scatter plotting using Microsoft Office Excel 2010 and the regression models were reported.

3. Results

3.1. Microbial stress factors in fermented mawè doughs

During fermentation, the pH dropped from 6.2 to 3.4 ± 0.1 in maize mawè and 6.2 to 3.7 ± 0.1 in sorghum mawè after 72 h of fermentation time (Table 2). Ethanol increased from 0.0% in both mawè doughs to 3.0 ± 1.1% (*v*/*v*) for maize mawè and 3.0 ± 0.1% (v/v) for sorghum mawè. Lactate increased from 25.7 ± 29.1 mM to 285.4 ± 19.2 mM in maize mawè and from 84.9 ± 34.6 mM to 171.1 ± 62.2 mM in sorghum mawè. Acetate increased from 2.5 ± 2.9 mM to 149.8 ± 23.6 mM in maize mawè and from 53.4 ± 16.2 mM to 90.6 ± 25.8 mM in sorghum mawè. For lactic acid and acetic acid, high variation was observed especially at the onset of the fermentation (standard deviations > 100) probably due to the high variation in the soaking time (6–14 h) used in the two production sites during the processing of the mawè doughs (Fig. S1) from where samples were picked. The highest concentrations of lactate (285 mM) and acetate (150 mM) were obtained in maize based-mawè dough after 72 h of fermentation whereas 3% (*v*/*v*) ethanol was recorded in both mawè doughs.

3.2. Effect of the stress conditions on maximum specific growth rate of the yeast strains

Table 3 shows the maximum specific growth rates (μ_{max}) of the yeast strains growing in non-stress condition, i.e. MYGP pH 5.6 as well as in the stress conditions. In non-stress condition, μ_{max} of *S. cerevisiae* strains was 0.32–0.35 h^{-1}, μ_{max} of *K. marxianus* strains was 0.31–0.33 h^{-1}, μ_{max} of *P. kudriavzevii* strains was 0.27–0.29 h^{-1} and μ_{max} of *C. glabrata* strains was 0.35–0.41 h^{-1}.

Exposed to pH 3.4, μ_{max} significantly decreased to 0.28–0.31 h^{-1} for *S. cerevisiae* strains ($P \leq 0.01$) and to 0.32–0.34 h^{-1} for *C. glabrata* strains Cg2 and Cg3 ($P \leq 0.05$). No significant decrease of μ_{max} occurred ($P > 0.05$) at pH 3.4 for *C. glabrata* strain Cg1 and for all *K. marxianus* and *P. kudriavzevii* strains. Exposure to 3% (*v*/*v*) ethanol ($EtOH_{pH\ 3.4}$) decreased μ_{max} for all *S. cerevisiae* (0.23–0.30 h^{-1}) and *C. glabrata* (0.28–0.30 h^{-1}) strains, while no decrease was recorded for two of the three *K. marxianus* strains (Km2 and Km3) and all the *P. kudriavzevii* strains. Exposure to 285 mM lactic acid ($LA_{pH3.4}$) significantly affected the growth of all yeast strains and μ_{max} was reduced to 0.13–0.15 h^{-1} for *S. cerevisiae*, to 0.18–0.21 h^{-1} for *K. marxianus*, to 0.11–0.14 h^{-1} for *P. kudriavzevii* and to 0.19–0.26 h^{-1} for *C. glabrata*. Furthermore, growth of all yeasts was fully inhibited and μ_{max} was 0.0 h^{-1} when the strains were exposed to 150 mM acetic acid (AA

Table 2
Changes in pH, ethanol and weak organic acids during spontaneous fermentations of two types of mawè doughs.

		Fermentation time					
		0 h	6 h	12 h	24 h	48 h	72 h
Commercial maize mawè	pH	6.2 ± 0.1^{a}	4.7 ± 0.1^{b}	4.3 ± 0.1^{c}	3.8 ± 0.1^{d}	3.5 ± 0.1^{e}	3.4 ± 0.1^{e}
	Ethanol (% *v/w*)	0.0 ± 0.0^{a}	0.0 ± 0.2^{a}	0.0 ± 0.3^{a}	0.3 ± 0.2^{a}	0.2 ± 0.2^{b}	3.0 ± 1.1^{b}
	Lactate (mM)	25.7 ± 29.1^{a}	107.7 ± 20.1^{b}	154.9 ± 10.5^{c}	204.8 ± 26.6^{d}	254.3 ± 29.6^{e}	285.4 ± 19.2e
	Acetate (mM)	2.5 ± 2.9^{a}	39.1 ± 45.6^{ab}	86.8 ± 30.3^{bc}	136.2 ± 11.2^{d}	143.1 ± 49.9^{d}	149.8 ± 23.6^{d}
Commercial sorghum mawè	pH	6.2 ± 0.1^{a}	4.5 ± 0.2^{b}	4.2 ± 0.1^{c}	3.9 ± 0.0^{d}	3.8 ± 0.0^{d}	3.7 ± 0.1^{d}
	Ethanol (% %)	0.0 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.1 ± 0.0^{a}	0.7 ± 0.0^{a}	3.0 ± 0.1^{b}
	Lactate (mM)	84.9 ± 34.6^{a}	130.3 ± 19.1^{ab}	119.9 ± 34.4^{ab}	181.1 ± 17.0^{b}	174.9 ± 67.3^{b}	171.1 ± 62.2^{b}
	Acetate (mM)	53.4 ± 16.2^{a}	61.8 ± 24.3^{ab}	58.5 ± 13.0^{a}	75.2 ± 12.8^{abc}	100.8 ± 22.3^{bc}	90.6 ± 25.8^{c}

Values presented correspond to means ± sd of four individual measures obtained for samples picked in duplicate from 2 different production sites for each type of mawè doughs. Values with the same letters within a line do not differ significantly (p > 0.05).

$_{pH\ 3.4}$) or the combination of stress conditions, i.e. (LA + AA)$_{pH\ 3.4}$ and (LA + AA + EtOH)$_{pH\ 3.4}$.

3.3. Viability of yeast strains upon exposure to stress conditions as determined by flow cytometry

Fig. 1 shows representative cytograms (density plots) obtained by flow cytometric analysis of the most resistant yeast strains of each species to the combination of stress conditions, i.e. (LA + AA + EtOH)$_{pH\ 3.4}$. The cytograms clearly shows four different subpopulations of cells for each strain. The subpopulation of viable cells with intact membrane stained with green fluorescence dye SYTO 13 (emission 530 nm) are detected in quadrant Q1.The subpopulation of dead cells with damaged membrane, permeable to red fluorescence dye PI (emission 692 nm) are detected in quadrant Q2. The subpopulation in quadrant Q3 comprises cells in the intermediate physiological state permeable to both SYTO 13 and PI. Weakly stained cells are detected in quadrant Q4. For all four examined yeast strains, the cells populations are rather homogeneous in non-stress condition at pH 5.6 after 6 h, i.e. 94.3–97.0% of the cells are recorded in Q1. Increased differences in sensitivities between the strains were observed at pH 3.4 (i.e. 86.8–95.0% of the cells were recorded in Q1), 3% (*v*/v) ethanol (EtOH$_{pH3.4}$) (i.e. 79.3–96.3% of the cells in Q1) and 150 mM acetic acid (AA$_{pH3.4}$) (i.e. 82.5–93.5% of the cells in Q1), whereas practically no differences in sensitivity were observed after 6 h in 285 mM lactic acid (LA$_{pH3.4}$) (i.e. 93.2–96.5% of the cells in Q1). Exposure for 6 h to the combinations of the stress conditions significantly influenced the viability of all yeast strains even though differences in sensitivities were observed among the yeast species, i.e. for (LA + AA)$_{pH\ 3.4}$ 2.6–53.9% of the cells were recorded in Q1 and for (LA + AA + EtOH)$_{pH\ 3.4}$ 0.1–39.0% of the cells were recorded in Q1.

The heatmap in Fig. 2 and Table S1 present the proportions of viable cells counted in quadrant Q1 for all twelve strains after exposure to the different stress conditions for 6, 24, 48 and 72 h as compared to the proportion of viable cells at the non-stress conditions. Both inter- and intraspecies variations were observed. Exposure to pH 3.4 for up to 48 h did not affect the proportion of viable cells for any of the strains, however, *S. cerevisiae* Sc2, *K. marxianus* Km1 and *P. kudriavzevii* Pk1 and Pk3 were affected at 72 h ($P < 0.05$). Exposure to EtOH$_{pH3.4}$ for up to 72 h did not affect the proportion of viable cells for any of the strains except for *P. kudriavzevii* Pk1 at 6, 24 h ($P < 0.05$) and 72 h ($P < 0.01$), *P. kudriavzevii* Pk2 and *C. glabrata* Cg2 at 72 h ($P < 0.05$). Exposure to LA$_{pH3.4}$ for up to 24 h did not affect the proportion of viable cells for any of the strains except for *S. cerevisiae* Sc3 and *K. marxianus* Km1 ($P < 0.05$). Exposure to LA$_{pH3.4}$ for 48 h and 72 h affected the proportion of viable cells of *S. cerevisiae* Sc1 ($P < 0.05$) and Sc3 ($P < 0.01$) and the three *K. marxianus* and *P. kudriavzevii* strains ($P < 0.01$). However, *C. glabrata* Cg1 and Cg3 were not affected, while Cg2 was affected only at 72 h ($P < 0.05$). Exposure to (AA$_{pH3.4}$) for up to 48 h did not affect the proportion of viable cells of any of the three *S. cerevisiae* strains. The proportion of viable cells were affected by exposure to AA$_{pH3.4}$ for two out of three *K. marxianus* (i.e. Km1 and Km3) and *C. glabrata* (i.e. Cg1 and Cg3) strains at 6 h, for all the three *K. marxianus* and *C. glabrata* strains at 24 h, 48 h and 72 h, while the three *P. kudriavzevii* strains were affected only at 48 h and 72 h. Exposure to the combined stress conditions i.e. (LA + AA)$_{pH\ 3.4}$ and (LA + AA + EtOH)$_{pH\ 3.4}$ for 6 h, 24 h, 48 h and 72 h affected the proportion of viable cells of all the yeast strains. Exposed to the combined stress condition

Table 3
Maximum specific growth rate (μ_{max}, h^{-1}) of yeast strains growing at non-stress condition i.e. MYGP (pH 5.6) and at the stress conditions [pH 3.4, 3% (*v*/v) ethanol (EtOH$_{pH3.4}$), total lactic acid 285 mM (LA $_{pH\ 3.4}$), total acetic acid 150 mM (AA $_{pH\ 3.4}$), the combinations of (LA + AA) $_{pH\ 3.4}$ and (LA + AA + EtOH)$_{pH\ 3.4}$; all stress conditions being adjusted to pH 3.4].

Strain	pH 5.6	pH 3.4	EtOH$_{pH3.4}$	LA $_{pH3.4}$	AA $_{pH3.4}$	(LA + AA)$_{pH3.4}$	(LA + AA + EtOH)$_{pH3.4}$
Sc1	0.35 ± 0.01	0.31 ± 0.00**	0.23 ± 0.11***	0.13 ± 0.07***	0.02 ± 0.07***	0.00 ± 0.00***	−0.00 ± 0.01***
Sc2	0.32 ± 0.01	0.28 ± 0.02***	0.24 ± 0.02***	0.15 ± 0.01***	0.01 ± 0.00***	0.01 ± 0.01***	0.01 ± 0.01***
Sc3	0.33 ± 0.01	0.31 ± 0.01***	0.30 ± 0.022**	0.15 ± 0.01***	−0.01 ± 0.01***	0.01 ± 0.08***	−0.02 ± 0.04***
Km1	0.31 ± 0.01	0.30 ± 0.01	0.28 ± 0.01**	0.21 ± 0.00***	0.07 ± 0.05***	0.03 ± 0.03***	0.04 ± 0.00***
Km2	0.33 ± 0.01	0.31 ± 0.01	0.32 ± 0.02	0.18 ± 0.05***	0.04 ± 0.04***	0.01 ± 0.00***	0.00 ± 0.01***
Km3	0.33 ± 0.01	0.32 ± 0.01	0.31 ± 0.01	0.19 ± 0.01***	0.03 ± 0.00***	0.02 ± 0.00***	0.02 ± 0.04***
Pk1	0.27 ± 0.02	0.24 ± 0.03	0.28 ± 0.04	0.12 ± 0.01***	0.01 ± 0.00***	0.07 ± 0.01***	−0.01 ± 0.02***
Pk2	0.28 ± 0.02	0.24 ± 0.02	0.27 ± 0.02	0.11 ± 0.01***	0.01 ± 0.01***	0.06 ± 0.01***	0.03 ± 0.03***
Pk3	0.29 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	0.14 ± 0.01***	0.03 ± 0.00***	0.07 ± 0.00***	0.07 ± 0.04***
Cg1	0.41 ± 0.03	0.39 ± 0.02	0.28 ± 0.03***	0.26 ± 0.11***	0.04 ± 0.11***	0.03 ± 0.06***	−0.02 ± 0.02***
Cg2	0.37 ± 0.03	0.34 ± 0.01*	0.30 ± 0.03***	0.24 ± 0.01***	0.04 ± 0.04***	0.04 ± 0.04***	0.06 ± 0.07***
Cg3	0.35 ± 0.01	0.32 ± 0.01**	0.30 ± 0.01***	0.19 ± 0.01***	−0.01 ± 0.00***	0.06 ± 0.09***	−0.01 ± 0.01***

The μ_{max} values were determined by fitting the growth curves using DMfit software (Institute of Food Research, Norwich, UK). The OD_{600} values were measured in 96-wells microplate every hour until 24 h. Values are mean ± sd for six individual measurements from two biological replicates. Within a line, means calculated for stress conditions were each compared to mean calculated for the non-stress condition using Student's *t*-test and asterisks (*, **, ***) denote statistically significant differences for α = 0.05, 0.01 and 0.001, respectively.

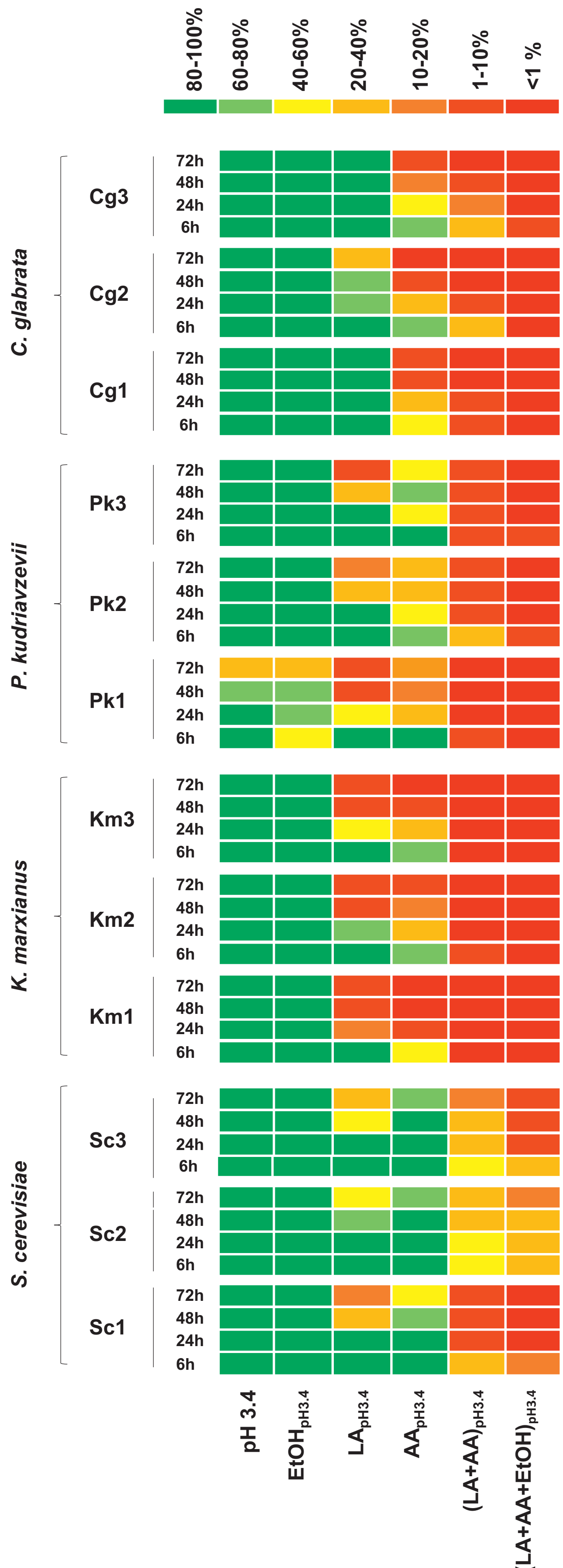


Fig. 2. Changes in viable cell proportion of yeast strains exposed to the stress conditions i.e. low pH (pH 3.4), 3% (*v*/v) ethanol (EtOH$_{pH3.4}$), 285 mM total lactic acid (LA$_{pH3.4}$), 150 mM total acetic acid (AA$_{pH3.4}$), and the combinations of these stress conditions (LA + AA)$_{pH\ 3.4}$ and (LA + AA + EtOH)$_{pH\ 3.4}$. The proportions are means calculated from two biological replicates using data of the density plot images from flow cytometric analysis.

mimicking fermented mawè dough, i.e. (LA + AA + EtOH)$_{pH\ 3.4}$ for 72 h, *S. cerevisiae* strains were the least sensitive (viable cell proportions were 0.1–15.2% after 72 h exposure) followed by *P. kudriavzevii* strains (viable cell proportions were 0.1–0.4% after 72 h exposure) while *K. marxianus* and *C. glabrata* strains were the most sensitive (viable cell proportions were 0.0% after 72 h exposure).

3.4. Growth and survival of yeast strains upon exposure to stress conditions as determined by plate counting

The flow cytometric results were confirmed by plate counting on MYGP, pH 5.6 after exposure to the non-stress or the six stress conditions for 6, 24, 48 or 72 h (Fig. 3). For all the yeast strains (except Pk1), growth was observed for non-stress condition (pH 5.6) and after exposure to pH 3.4 and 3% (*v*/v) ethanol (EtOH$_{pH3.4}$) at all time points, i.e. 6–72 h where cell counts increased by a factor of ≤1.0 log at 6 h and 1.0–2.0 log at 24, 48 and 72 h. For all the yeast strains, growth was observed at 285 mM lactic acid, pH 3.4 (LA$_{pH3.4}$) at 24 h with cell counts increased by a factor < 1.0 log (except for Cg1 and Cg2 with cell counts increased by a factor 1.0–1.3 log), however, the cell counts remained at this level ($P > 0.05$) at 48 and 72 h for *S. cerevisiae* and *C. glabrata* strains but decreased significantly ($P < 0.05$) to < 1.0 log for two out of three *P. kudriavzevii* strains (i.e. Pk2 at 48 h and Pk3 at 48 and 72 h). Moreover, growth inhibition with log reductions < 0.5 log were seen for all *K. marxianus* strains and *P. kudriavzevii* Pk1 at 48 and 72 h.

For all yeast strains, growth inhibition was seen at 150 mM acetic acid, pH 3.4 (AA$_{pH3.4}$) and at the combined stress conditions, i.e. (LA + AA)$_{pH\ 3.4}$ and (EtOH + LA + AA)$_{pH\ 3.4}$ at all-time points, i.e. 6–72 h of exposure. Exposure to (AA$_{pH3.4}$) for 72 h caused reductions of 0.3–0.5 log for all *S. cerevisiae* strains and reductions of 0.5–0.9 log for all *P. kudriavzevii* strains, while reductions of 1.0–3.0 log were seen for all *C. glabrata* and *K. marxianus* strains. After 72 h exposure to (LA + AA)$_{pH\ 3.4}$, the reductions were 1.0–2.0 log for all *S. cerevisiae* strains and *P. kudriavzevii* Pk2 and Pk3, 2.3 log for Pk1, > 3 log for *C. glabrata* Cg1 and Cg2 and 1.9 log for Cg3, while all *K. marxianus* strains had reduction of 6.7 log at 48 h and 72 h of exposure. For (EtOH + LA + AA)$_{pH\ 3.4}$ the reduction after 72 h were < log 2.0 for *S. cerevisiae* Sc2 and Sc3 and slightly higher for Sc1 (2.6 log). Reductions of 6.7 log were recorded at all time points, i.e. 6–72 h for all *K. marxianus* strains. For all *P. kudriavzevii* strains reductions of 3.0–4.0 log were recorded at 72 h, while reductions of 6.7 log were recorded at 48 h and 72 h for *C. glabrata* Cg1 and Cg2, and at 72 h for Cg3.

3.5. Ability of yeast cells to recover from stress damages

The individual cells of one stress resistant *S. cerevisiae* Sc2 and one stress sensitive *K. marxianus* Km1 were investigated for their ability to recover from stress damages caused by the combination of stress conditions occurring in the mawè dough at 72 h of fermentation, i.e. (EtOH + LA + AA)$_{pH\ 3.4}$. Examples of corresponding brightfield and PI fluorescent images of stressed cells of Sc2 and Km1 after transfer to non-stress condition, i.e. flushing with MYGP broth, pH 5.6 are shown in Fig. 4. Dividing cells were recorded only for the stress resistant *S. cerevisiae* Sc2, whereas no cells of the stress sensitive *K. marxianus* (Km1) showed proliferation within the 24 h after transfer to non-stress condition. Fig. 5 shows the distribution of single cells of the stress resistant *S. cerevisiae* Sc2 and stress sensitive *K. marxianus* Km1 according to the

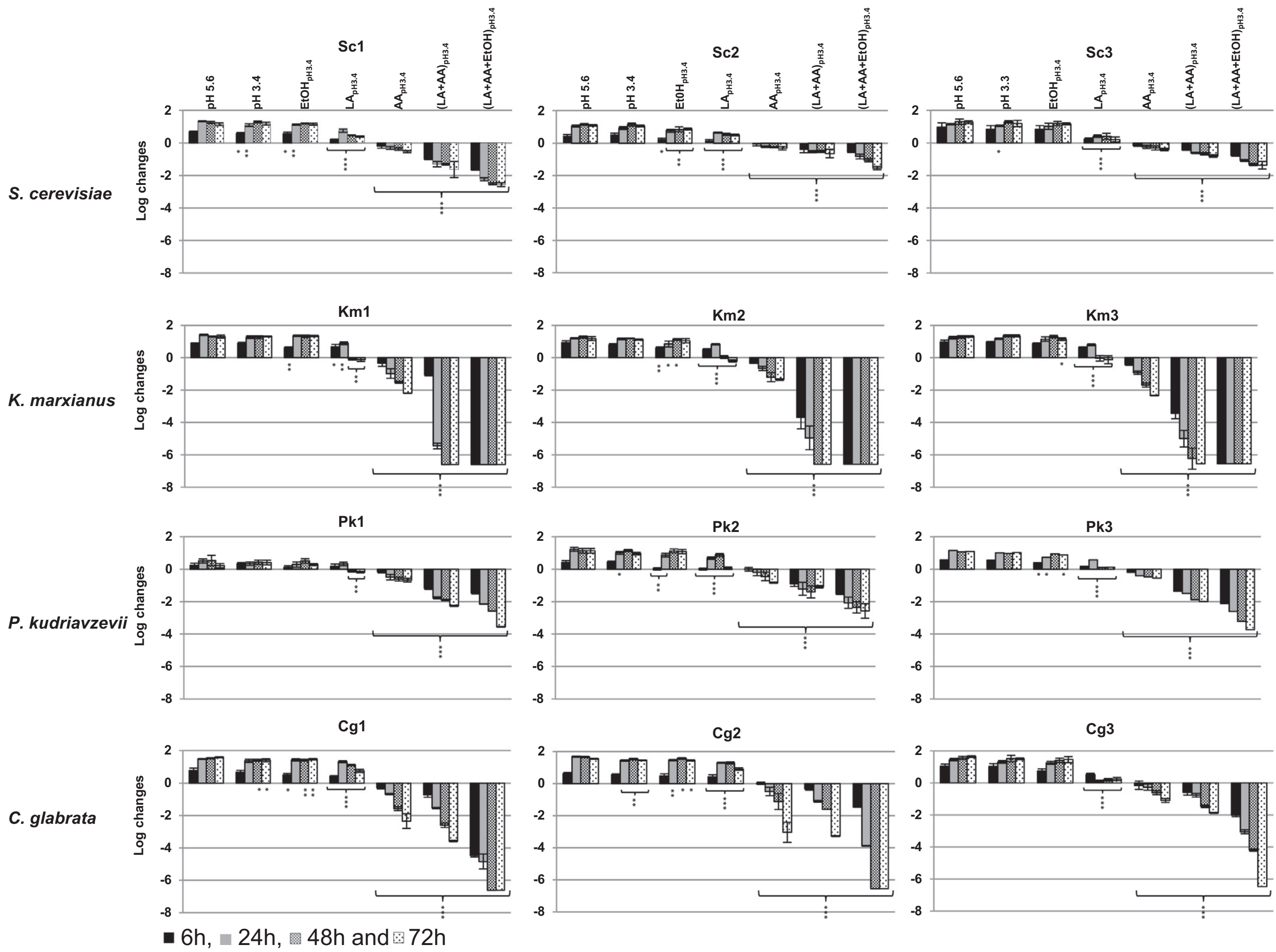


Fig. 3. Log changes of yeast species exposed to non-stress condition (pH 5.6), and the stress conditions i.e. low pH (pH 3.4), ethanol 3% (*v*/v) ($EtOH_{pH3.4}$), 285 mM total lactic acid ($LA_{\ pH\ 3.4}$), 150 mM total acetic acid ($AA_{\ pH\ 3.4}$), and the combination stress conditions $(LA + AA)_{pH\ 3.4}$ and $(LA + AA + EtOH)_{pH\ 3.4}$. Samples were analyzed after 6 h, 24 h, 48 h and 72 h by streaking on MYGP agar, pH 5.6 and incubation at 25 °C for 2–3 days. The log values were calculated using CFU values. The mean and SD (bars) were calculated from two biological replicates. Means for stress conditions were each compared to non-stress condition using Student's *t*-test. Asterisks (*, **, ***) denote statistically significant differences for α = 0.05, 0.01 and 0.001 respectively.

duration of the lag phase and pH_i, upon transfer from combined stress condition $(EtOH + LA + AA)_{pH\ 3.4}$ to non-stress condition, by flushing with MYGP broth, pH 5.6 for 24 h. Of the stress resistant *S. cerevisiae* Sc2 cells, 37.9 ± 10.8% resumed proliferation after 3–12 h in non-stress condition and were defined as having short to medium lag phase. These cells re-adjusted their pH_i to near physiological range (6.0–7.4) after 30 min in non-stress condition with no significant change after 90 min in non-stress condition. Long lag phases were observed for 7.3 ± 5.1% of the Sc2 cells, which resumed proliferation after 12–24 h of flushing with MYGP broth, pH 5.6. Near physiological pH_i (6.0 (5.1–7.4) were recorded for the majority of these cells after 30 min in non-stress condition with no significant change after 90 min. After 24 h in non-stress condition 53.5 ± 6.3% of the Sc2 cells and none of the stress sensitive *K. marxianus* Km1 cells were able to proliferated upon transfer from the combined stress condition $(EtOH + LA + AA)_{pH\ 3.4}$ to non-stress condition by flushing with MYGP broth, pH 5.6. For these non-proliferating cells of Sc2, lower pH_i (5.1–6.0) was recorded for 22.9 ± 5% and acidic pH_i (≤5.0) for 30.6 ± 4%, after 30 min in non-stress conditions and 90 min in non-stress condition. For the non-proliferating cells of Km1, acidic pHi (≤5.0) was recorded for 97.9 ± 1.2% after 30 min in non-stress condition with significant decrease (*P = 0.04*) after 90 min (92.1 ± 0.4%). The non-proliferating cells of both Sc2 and Km1 strains displayed PI fluorescence after 24 h in non-stress condition, indicating that these cells were dead.

3.6. Comparison and correlation between methods in yeast viability assessment

The scatterplots in Fig. 6 shows the correlation coefficients (R^2) at strain and species levels between ratios of viable cells as determined by flow cytometry and plate counting, respectively. At strain level, the R^2 values varied between 0.97 and 0.99 for the twelve investigated yeast strains. At species level, the R^2 values were slightly higher for *K. marxianus* ($R^2_{Km} = 0.98$), *C. glabrata* ($R^2_{Cg} = 0.98$) and *S. cerevisiae* ($R^2_{Sc} = 0.97$) compared with *P. kudriavzevii* ($R^2_{Pk} = 0.93$). This result indicated high variation between *P. kudriavzevii* strains. The slopes of the correlation curves calculated at species level show that plate counting seemed to generally underestimate the proportion of viable cells as compared to flow cytometry, however, species variations were observed. As seen from the correlation curves of the scatterplots, only 68% of the viable cells detected by flow cytometry were culturable for *S. cerevisiae*, 67% for *K. marxianus* and 71% for *P. kudriavzevii,* whereas 99% of the viable *C. glabrata* cells, as determined by flow cytometry, were culturable.

Fig. 7. shows the proportion of cells of *S. cerevisiae* Sc2 and *K. marxianus* Km1 determined as viable, in intermediate state or dead by

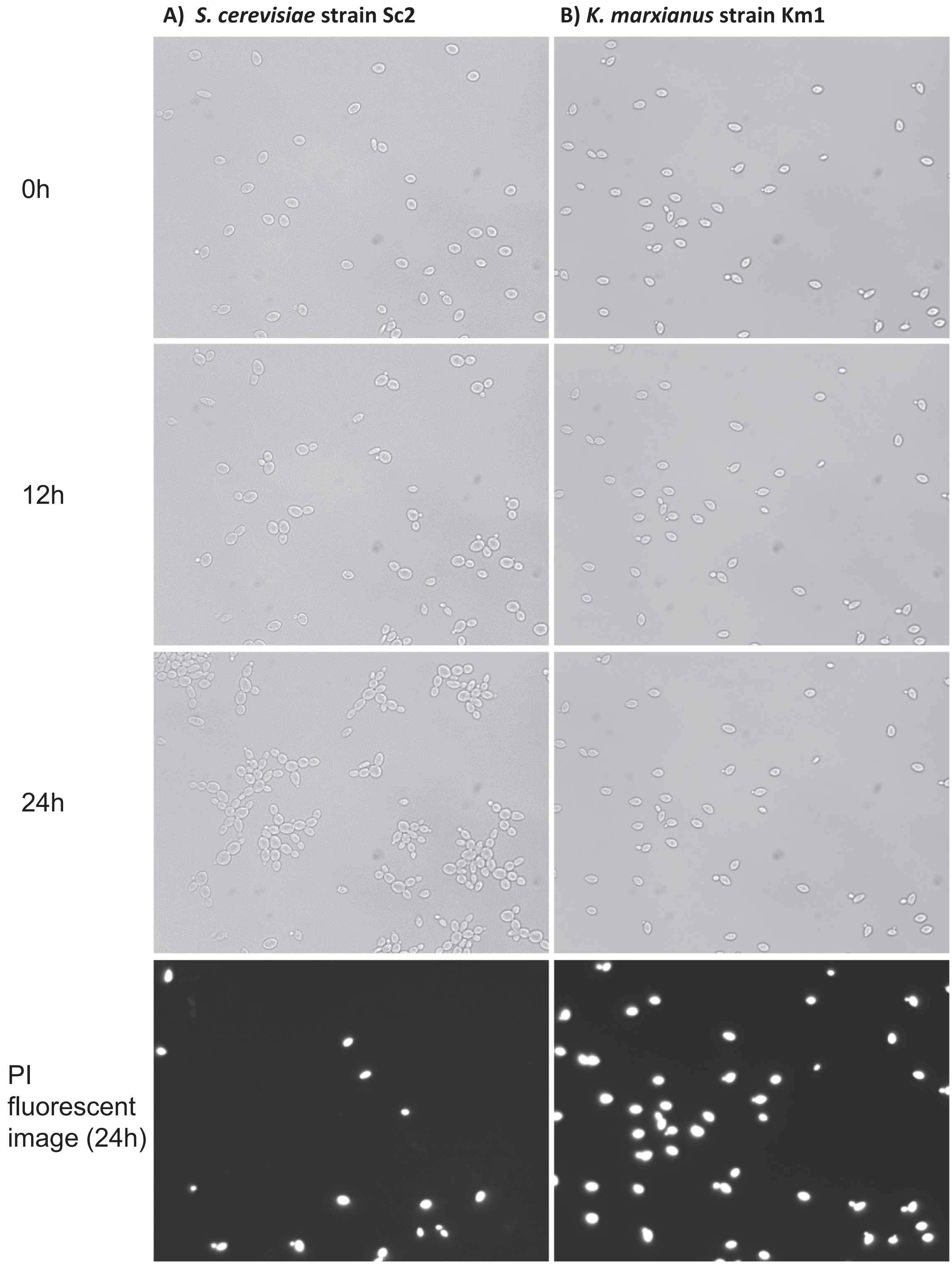


Fig. 4. Proliferation of stressed cells of A) the most resistant strain (*S. cerevisiae* Sc2) and B) the most sensitive strain (*K. marxianus* Km1) transferred to non-stress condition by flushing with MYGP broth, pH 5.6 over 24 h in CellASIC ONIX Y04C microfluidic yeast plate. The cells were stressed for 6 h in the combined stress condition i.e. combination of 285 mM total lactic acid, 150 mM total acetic acid and 3% (*v*/v) ethanol at pH 3.4. The brightfield images were recorded over 24 h of incubation. At 24 h of incubation, the cells were stained by flushing with PI solution and the fluorescence images were recorded.

flow cytometric analysis, fluorescence microscopy analysis or plate counting. Interestingly, the proportion of viable cells (35.9 ± 4.5%) with intact plasma membrane of Sc2 cells counted in quadrant Q1 of the flow cytometric cytograms was similar to the proportion (37.9 ± 10.8%) of non-PI fluorescent cells with short to medium lag phase (3 − 12 h) with pH_i 6.0–7.4, as determined in fluorescent microscopy. These proportions were higher, but not significant ($P = 0.17$), to the culturable proportion (20.6 ± 3.8%) of Sc2 cells. Intermediate cell proportion (8.1 ± 5.2%) of Sc2 recorded in quadrant Q3 of the flow cytometric cytograms was similar to the proportion (7.3 ± 5.3%) of

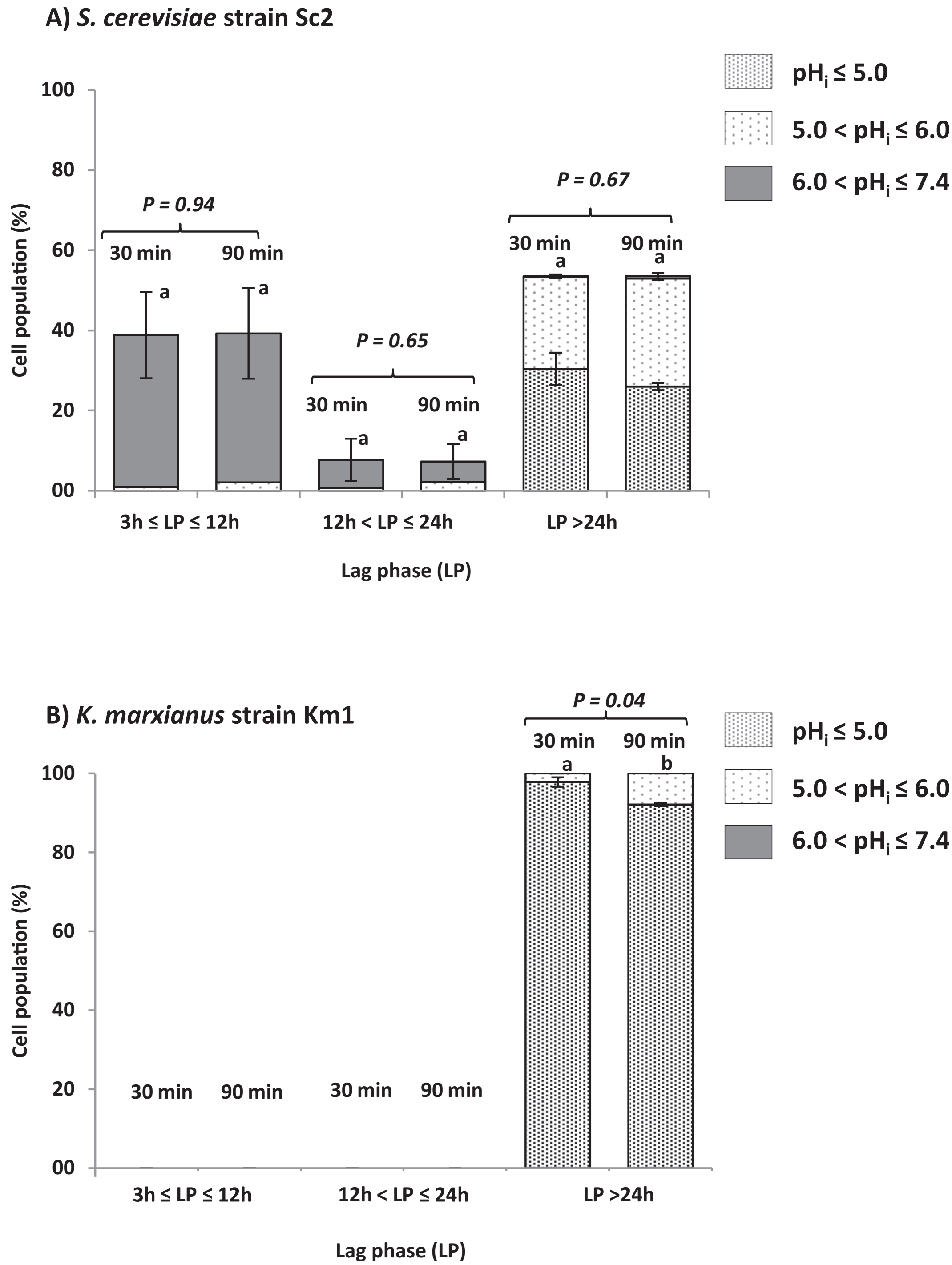


Fig. 5. Distribution of single cells of A) the most resistant strain (*S. cerevisiae* Sc2) and B) the most sensitive strain (*K. marxianus* Km1) according to their intracellular pH (pH_i) and lag phase i.e. time required to resume proliferation in non-stress condition (MYGP, pH 5.6). The cells were stressed for 6 h in the combined stress condition i.e. combination of 285 mM total lactic acid, 150 mM total acetic acid and 3% (*v*/v) ethanol at pH 3.4. The stressed cells were transferred in non-stress condition and pH_i was analyzed after 30 min and 90 min and the time required for individual cell to resume proliferation (lag time) was recorded. The proportions are mean of three biological replicates and error bars indicate the standard deviation. Means with the same letters do not differ significantly ($p > 0.05$).

cells exhibiting long lag phase (12–24 h) with pH_i 6.0–7.4, recorded by fluorescent microscopy. Dead cell proportion (54.7 ± 9.8%) of Sc2 recorded in quadrant Q2 of flow cytometric cytograms was similar to the proportion (53.5 ± 6.3%) of non-growing cells with PI fluorescence and $pH_i < 6$ recorded by fluorescent microscopy. However, the dead cell proportions as measured by flow cytometry and fluorescent microscopy were significantly lower than the non-growing cell proportion (79.4 ± 3.8%) of Sc2 as observed by plate counting. All three methodologies proved that practically all cells of Km1 were dead even though a low fraction (i.e. 5.2 ± 5.4%) of cells were determined as intermediate state by flow cytometry.

4. Discussion

For each of the twelve yeast strains exposed to the six different stress conditions, the cytograms obtained by flow cytometric analyses

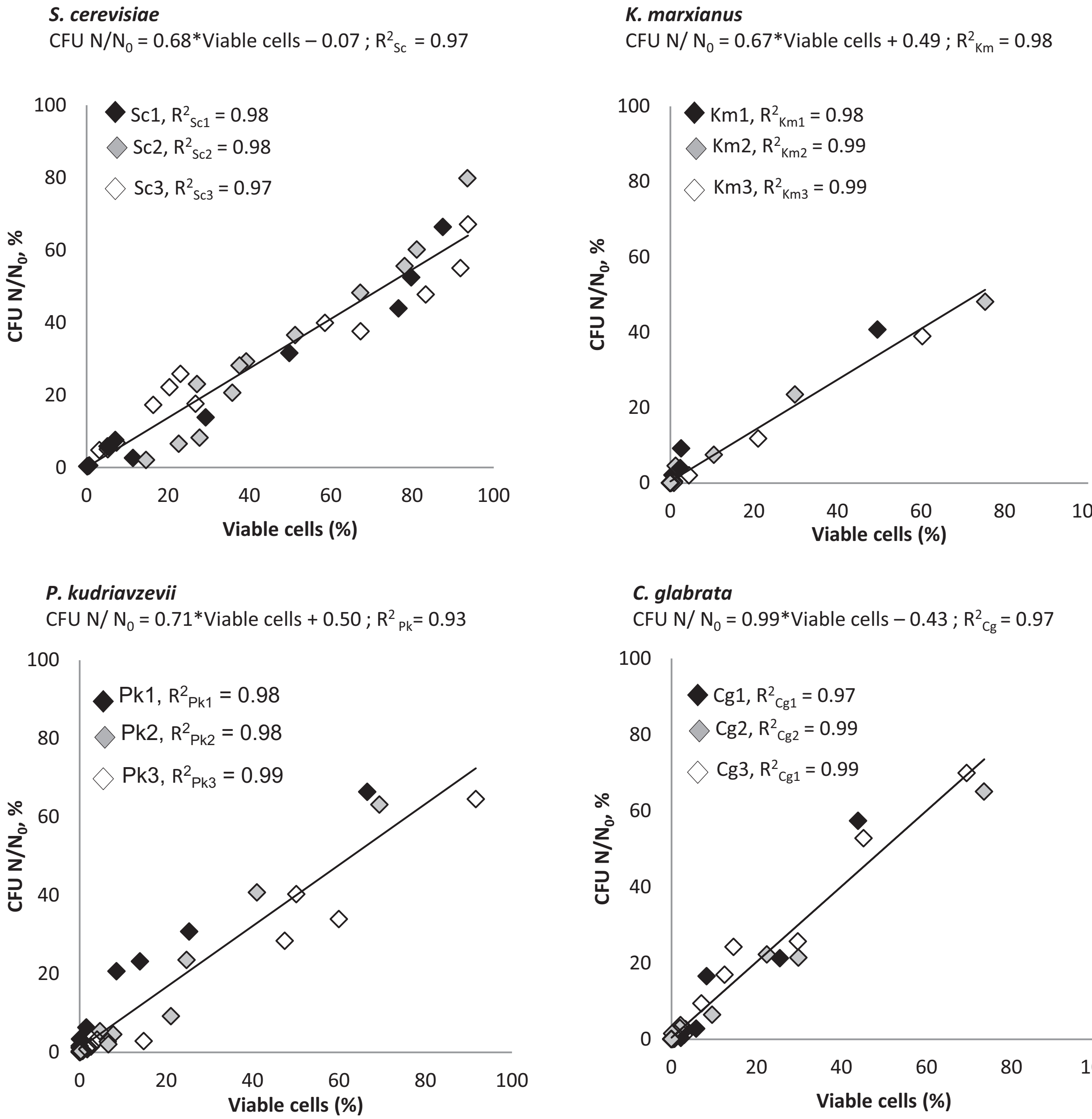


Fig. 6. Scatterplots showing statistical relationship between % viable cells determined by flow cytometry and plate counting (CFU N/No, %) of yeast strains exposed to 150 mM total acetic acid ($AA_{pH\ 3.4}$), and the combinations of the stress conditions $(LA + AA)_{pH\ 3.4}$ and $(LA + AA + EtOH)_{pH\ 3.4}$. For each point of the scatterplots, the CFU values and the viables cells values are determined for the same samples and the same time-points. The correlation coefficients R^2 were calculated at species and strain level using Pearson statistic with $P \leq 0.001$ for all correlations. The correlation curves were determined at species level and the significance of the slopes was determined for all the correlation curves with $P \leq 0.001$.

showed that PI-SYTO 13 dual staining allows high discrimination between viable cells with intact membrane, membrane damaged cells and dead cells. This discrimination is due to differences in cell membrane permeability to the fluorescent dyes as reported previously for bacterial viability assessment (Larsen et al., 2018; Zhao et al., 2017). Our results demonstrated that the differences in membrane permeability depend on the type of stress and the duration of exposure to stress conditions, and varies from species to species, from strain to strain and even from cell to cell. This result shows that cells from the same strain may react differently to stress conditions as previously reported for lactic acid bacteria (Zhao et al., 2017) and for *S. cerevisiae* (Guldfeldt and Arneborg, 1998). In general, the cell populations were homogeneous at pH 3.4 and 3% (*v*/v) ethanol ($EtOH_{pH3.4}$). However, depending on the exposure times and the sensitivity of the strain, high heterogeneity was observed at 285 mM lactic acid ($LA_{pH3.4}$), 150 mM acetic acid ($AA_{pH3.4}$) and at the combinations of the stress conditions i.e. $(LA + AA)_{pH\ 3.4}$ and $(LA + AA + EtOH)_{pH\ 3.4}$. Similarly, heterogeneity in cell populations has been reported for *S. cerevisiae* upon exposure to 96 mM acetic acid at pH 4.5, resulting in subpopulations with different intracellular pH values (Valli et al., 2005).

The results showed that the twelve yeast strains generally were resistant towards low pH 3.4 and $EtOH_{pH3.4}$. However, growth inhibition and/or decrease of proportions of viable cells were seen for all yeast strains at $LA_{pH3.4}$ and $AA_{pH3.4}$. Similar results have been reported for *S. cerevisiae* by Narendranath et al. (2001a, 2001b) who reported minimum inhibitory concentrations of lactic acid (278 mM) and acetic acid (100 mM) at pH 4.5 in minimal media. In the present study, combination of 285 mM lactic acid and 150 mM acetic acid at pH 3.4, i.e. $(LA + AA)_{pH\ 3.4}$ exerted a higher effect on viable cell proportions for all yeast strains compared to exposure to either lactic acid or acetic

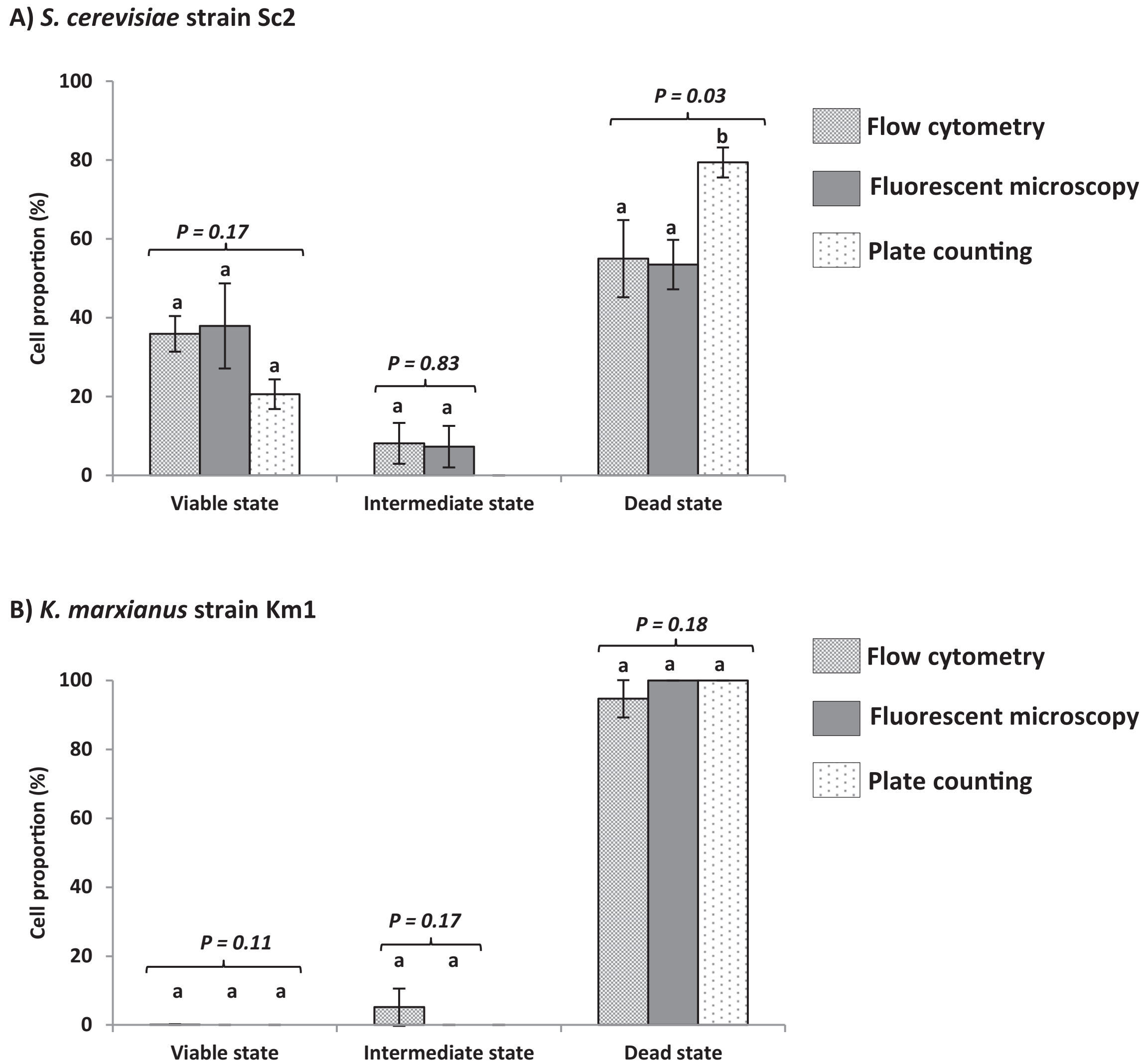


Fig. 7. Proportion of viable cells, intermediate state cells and dead cells as analyzed by flow cytometry (FC), fluorescent microscopy (FM) and plate counting (PC). Proportion of viable state is cells counted in quadrant Q1 (FC), with lag phase 3–12 h and pH_i 6.1–7.4 (FM) being able to grow on plate, MYGP, pH 5.6 (PC). Proportion of intermediate state is cells counted in quadrant Q2 (FC) and with lag phase 12–24 h and pH_i 5.1–6.0 (FM). Proportion of dead state is cells counted in quadrant Q3 (FC), with no proliferation at 24 h and pH_i 5.1–6.0 (FM) and cells that did not grow on plate (PC). The proportions are means calculated from two biological replicates and error bars indicate the standard deviation. Means with the same letters do not differ significantly ($p > 0.05$).

acid. This synergetic inhibitory effect of lactic- and acetic acids on yeast growth has been previously reported (Narendranath et al., 2001a). Addition of 3% (*v*/v) ethanol to $(LA + AA)_{pH\ 3.4}$ increased dramatically the damaged cell and dead cell proportions. Ethanol concentration of 3% (*v*/v) may increase the fluidity of the plasma membrane and may weaken or destroy the normal membrane structures (Ding et al., 2009; Teixeira et al., 2009), thereby enhancing the entry of undissociated weak organic acids into the cytosol. The sensitivity of yeasts species at stress conditions occurring in mawè dough at the late stages of fermentation, as investigated in the present study, was performed in liquid culture while cereal dough is a solid matrix. The solid state matrix could also enhance the toxicity of the cereal dough stress factors on yeast survival as the solid state fermentation involves growth of microorganisms on moist solid substrates in the absence of or limitation of free flowing water (Raghavarao et al., 2003).

In the present study *S. cerevisiae* strains were determined as the most resistant to the combination of stress conditions, i.e. $(LA + AA + EtOH)_{pH\ 3.4}$ mimicking the combined stress condition of fermented mawè dough. The second most resistant species was *P. kudriavzevii* followed by *K. marxianus* and *C. glabrata*. In earlier studies, *P. kudriavzevii* was previously determined as more resistant to 106.4 mM undissociated lactic acid at pH 2.5 compared to *S. cerevisiae* (Halm et al., 2004). Other studies have similarly reported that different strains of *P. kudriavzevii* had higher specific growth rate at low pH (Toivari et al., 2013) and higher tolerance to 2.5 g/L i.e. 41.6 mM total acetic acid (Ruyters et al., 2015) as compared to strains of *S. cerevisiae*, indicating that *P. kudriavzevii* is more tolerant to some stress conditions. In the present study, differences in sensitivity towards the stress conditions between strains of the same species was observed, which was especially apparent for *P. kudriavzevii*. Likewise, differences in acetic acid tolerance has been reported for different *S. cerevisiae* strains (Fernández-Niño et al., 2015). Moreover, differences in sensitivity between and within yeast species and strains towards the combined stress condition corresponding to the late stages of dough fermentation, i.e. $(LA + AA + EtOH)_{pH\ 3.4}$ may be due to unequal ability to maintain membrane integrity and as such intracellular pH (pH_i). As previously reported, the ability of yeast cells to maintain pH_i at different physiological conditions is fundamental to maintain viability and to regain culturability after removal of the stress (Orij et al., 2011; Ullah et al., 2013b). Our results demonstrated that only cells not being stained with PI (membrane integrity) or being culturable were able to maintain their pH homeostasis. Hence, the results showed that pH_i and plasma membrane integrity are inter-related physiological parameters, which together greatly influence yeast cell viability and culturability. The results confirm that pH_i is a tightly regulated physiological parameter in yeast cellular systems as previously reported (Orij et al., 2011).

Growth measurement by plate counting provided information on yeast sensitivity to stress at population level but failed to give insights into single cell physiology and cell population heterogeneity. Contrary, viability assessment by flow cytometry gave additional understanding on population heterogeneity, including viable, damaged, and dead subpopulations in few hours. Moreover, cell proliferation, membrane permeability and pH_i determination by fluorescence microscopy provided results in near real time regarding plasma membrane integrity, culturability and pH_i of single cells. Similarly, previous studies have reported flow cytometry and fluorescence microscopy methods as powerful techniques for rapid analysis of single cells (Díaz et al., 2010; Larsen et al., 2018; Zhao et al., 2017). Though results from flow cytometry and plate counting were highly correlated, plate counting slightly underestimated yeast viability for three of the four species. The combined approach of flow cytometry and plate counting helped to estimate the viable but non culturable (VBNC) cell proportion in yeast species. This combined approach has likewise successfully been used to assess viability and culturability of yeast cells growing in sulfite stress (Serpaggi et al., 2012). Our results demonstrated that the use of fluorescent dyes, i.e. dual PI-SYTO 13 staining, for flow cytometry and PI staining for membrane integrity assessment combined with CFDA-se staining for pH_i determination with fluorescence microscopy is fast, accurate and reliable methodologies for assessment of yeast physiology and viability.

The low sensitivity of *S. cerevisiae* strains towards intrinsic stress conditions occurring in spontaneously fermented cereals offers interesting opportunities for the fermented cereal food sector, as this yeast species is generally regarded as safe and finds wide applications in many types of fermentation processes including other cereal fermentation as e.g. sourdough (De Vuyst et al., 2016; Hayford and Jespersen, 1999; Jespersen, 2003). However, *K. marxianus* also being a preferred yeast species and involved in many fermented cereal foods (Greppi et al., 2016; Houngbédji et al., 2018; Pedersen et al., 2012), is highly sensitive towards the intrinsic stress factors in the dough. This is in agreement with previous findings demonstrated that *K. marxianus* is more abundant at the initial stages of cereal dough fermentations while *S. cerevisiae* is more abundant at later stages (Houngbédji et al., 2018). There might be a need for optimizing the fermentation process to change the environment of fermented mawè dough in favor of *K. marxianus* as well as other less resistant *S. cerevisiae* strains, while the survival of opportunistic *C. glabrata* strains should be inhibited. According to the present results, long time exposure to the combined stress conditions did not inhibit growth of *S. cerevisiae* to the same extent as of *C. glabrata*, while full inhibition of *K. marxianus* was observed. In the present study, *P. kudriavzevii* showed less sensitivity to the combined stress conditions compared to *K. marxianus* and *C. glabrata*. *P. kudriavzevii* was found as the most abundant yeast in cereal fermentation in West Africa (Houngbédji et al., 2018; Hounhouigan et al., 1994). This species has been shown to enhance folate content and to produce phytase thereby enhance the bioavailability of micronutrients (Greppi et al., 2016). However, multidrug resistance of *P. kudriavzevii* has been reported (Yadav et al., 2012). Positive and negative aspects of *P. kudriavzevii* in West African fermented doughs need therefore to be further investigated.

Additional experiments will be relevant for investigating the interactions between the different yeast species and the lactic acid bacteria naturally being part of the indigenous microbiota of fermented cereal dough. Based on the interactions with preferred yeast species, beneficial homo- and heterofermentative lactic acid bacteria could be used together with yeasts as defined starter cultures. Predictive modeling of the fermentation process could be used to control the growth of preferred microorganisms. Such modeling must include growth and inhibition of opportunistic pathogens, i.e. *C. glabrata* at concentrations of weak organic acids and ethanol, pH kinetic, fermentation temperature and duration relevant for West African fermented cereal dough. This could help to establish the exact pH, concentrations of weak organic acids and ethanol that favor survival of preferred yeasts and in parallel, inhibit survival of opportunistic pathogenic yeasts during cereal dough fermentation.

This study is the first investigating how the combined stress factors in fermented mawè dough, i.e. low pH, ethanol, lactic acid, acetic acid, influence viability and physiological conditions of strains of predominant yeast species, i.e. *S. cerevisiae*, *K. marxianus*, *P. kudriavzevii* as well as the opportunistic pathogen *C. glabrata*. The use of fluorescent dyes in a combined approach with flow cytometry and fluorescent microscopy provided qualitative and quantitative information in near real time on the sensitivity between and within species and strains, physiological changes in membrane integrity and pH_i of single cells. The sensitivity of the examined yeasts towards the stress condition of fermented mawè dough was species, strain, and cell dependent. Hence, all cells of the three *K. marxianus* strains and the three *C. glabrata* strains failed to withstand the combined stress conditions of mawè dough for 72 h indicating their high sensitivity to stress conditions occurring at the later stages of fermentation. Strains of *P. kudriavzevii* and especially strains of *S. cerevisiae* were less sensitive to the intrinsic stress factors in the mawè dough. The study point to the fact that further upgrading of the production of mawè dough is required focusing on strain selection for starter culture production. Studies including homo/hetero-fermentative lactic acid bacteria focusing on their acidification rate and production of weak organic acids should be conducted in order to get a full overview on the fermentation process as well as their influence on species and strain succession thereby favor preferable yeasts species and strains within e.g. *S. cerevisiae* and *K. marxianus* and to inhibit opportunistic pathogenic yeasts as e.g.. *C. glabrata.*

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