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Effect of mixed *Saccharomyces cerevisiae* Y10 and *Torulaspora delbrueckii* Y22 on dough fermentation for steamed bread making

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ABSTRACT

The use of non-*Saccharomyces* yeasts in dough fermentation has become increasingly popular because of their effects on product composition, texture and flavour. These yeasts are co-cultured with *Saccharomyces cerevisiae*. In this study, the characteristics of dough fermentation with combined *Torulaspora delbrueckii* Y22 and *S. cerevisiae* Y10 were investigated. In the dough containing co-cultures, *S. cerevisiae* Y10 cell populations increased rapidly and reached approximately 8.5 Log CFU/g wet dough, which is comparable to the monoculture after 24 h of fermentation. However, the cell number of *T. delbrueckii* Y22 did not significantly change throughout the dough fermentation ($p > 0.05$). When co-culture was used, the gas holding capacity and CO_2 production profile of the dough improved, and a high maltose concentration reaching 5.93 mg/g dry dough was observed after 12 h of dough fermentation. The mixed inocula of *S. cerevisiae* Y10 and *T. delbrueckii* Y22 enhanced the production of succinic acid, acetic acid and essential amino acids in the fermented dough. These results revealed the synergistic behaviour between *S. cerevisiae* Y10 and *T. delbrueckii* Y22 during dough fermentation and suggested the potential use of mixed yeast cultures in dough fermentation for steamed bread making.

1. Introduction

Steamed bread is a traditional staple food in China that is generally formulated with wheat flour, water and sourdough/Jiaozi/yeast (Li et al., 2016; Yeh et al., 2009; Zhu, 2014). Domesticated *Saccharomyces cerevisiae*, also known as baker's yeast, can produce high amounts of gas and is widely used in dough fermentation for steamed bread making because of its simplicity and short processing time (De Vuyst et al., 2016; Jayaram et al., 2013; Rezaei et al., 2014). The use of selected *S. cerevisiae* strains helps control the dough fermentation process and reduces the risk of spoilage. However, this method decreases the diversity of microflora and produces bread that lacks distinctive characteristics, such as unique texture, taste and flavour, compared with sourdough or Jiaozi fermentation (Keeratipibul et al., 2013; Wang et al., 2018; Wu et al., 2012).

Sourdough or Jiaozi fermentation always occurs in the presence of complex microbial consortia (Luangsakul et al., 2009; Wang et al., 2018; Zhang et al., 2011). The role of lactic acid bacteria (LAB) in sourdough fermentation has been extensively investigated (Alfonzo et al., 2016; Gobbetti et al., 2016), but few studies have focused on the role of yeast in improving dough fermentation and product quality (De Vuyst et al., 2016). Microbial communities in fermented dough consist of *S. cerevisiae* and high levels of non-*Saccharomyces* yeast species (De Vuyst et al., 2016; Li et al., 2016; Wang et al., 2018; Zhang et al., 2011). These yeasts may originate from starters, flour and water (Corona et al., 2016; Wang et al., 2018). The potential application of non-*Saccharomyces* yeasts in dough fermentation has been recently revealed (Aslankoohi et al., 2016; Daniel et al., 2011; Zhou et al., 2017). However, most non-*Saccharomyces* yeasts present poor gas production and can not complete dough fermentation (Aslankoohi et al., 2016; Liu et al., 2018). Therefore, the use of mixed inocula containing both non-*Saccharomyces* yeast and *S. cerevisiae* can be developed as an attractive biotechnological solution (Wahyono et al., 2016). However, the inappropriate use of non-*Saccharomyces* combined with *S. cerevisiae* may result in a series of fermentative problems, for instance, slow fermentation, off-flavours and lack of reproducibility (Liu et al., 2016). Using mixed inocula containing both non-*Saccharomyces* and *S. cerevisiae* can be also an attractive biotechnological solution that combines the advantages of recovering features from Jiaozi or sourdough fermentation with a strict control of the process. Thus, the dough fermentation features of the mixed yeasts and the overall taste and quality of the final product must be studied.

Torulaspora delbrueckii is one of the most abundant non-*Saccharomyces* species present in homemade corn and rye bread dough

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(Almeida and Pais, 1996; Pacheco et al., 2012) and appear highly represented in Jiaozi (Li et al., 2016). *T. delbrueckii* is also found on grape surface, and its strains show variation in their ability to ferment and assimilate carbon compounds (van Breda et al., 2013). *T. delbrueckii* has been previously assessed for its possible contribution to dough fermentation and bread making and for its good gas yield and satisfactory flavour as compared with non-*Saccharomyces* yeasts (Aslankoohi et al., 2016; Hernandez-Lopez et al., 2003). The biotechnological interest in *T. delbrueckii* has recently increased because of its high freezing and osmotic tolerance (Almeida and Pais, 1996; Hernandez-Lopez et al., 2003; Pacheco et al., 2012). Therefore, using *T. delbrueckii* in dough fermentation may be one of the most easily applied and inexpensive approaches for steamed bread makers to improve product quality and develop frozen dough technology.

S. cerevisiae strain Y10 and *T. delbrueckii* strain Y22 were isolated from Jiaozi (Li et al., 2016). To gain insights into the behaviour and contribution of *T. delbrueckii* Y22 in dough fermentation for steamed bread making, we evaluated the performance of controlled cultures of *S. cerevisiae* Y10 and *T. delbrueckii* Y22 in these fermentations. The dynamic changes of the two yeast species, the CO_2 production, the carbohydrate utilisation and the organic acid production were studied in co-culture and compared with each monoculture.

2. Materials and methods

2.1. Flour and microorganisms

Commercial wheat flour (0.38% ash, 10.89% protein and 13.79% moisture) was obtained from Jinyuan Flour Co., Ltd. (Zhengzhou, China).*S. cerevisiae* Y10 and *T. delbrueckii* Y22 were isolated from Jiaozi, a traditional starter for dough fermentation (Li et al., 2016). Both strains were sub-cultured at a 6-month interval on yeast extract peptone dextrose (YPD) agar medium, which contains 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 18 g/L agar and was maintained at 4 °C. The yeast cells were grown in YPD medium on a rotary shaker at 28 °C and harvested at the early stationary phase. Biomass was collected through centrifugation at 3000 r/min for 5 min (Sangon Biotech DM0412, China), washed and suspended in sterile water. The prepared yeast cells were immediately used for dough preparation.

2.2. Dough preparation and fermentation

Wheat dough was prepared as previously described but with modifications (Paramithiotis et al., 2006) as shown in Fig. 1. In brief, 300 g of flour and 135 mL of water were mixed in a mixing machine (Hauswirt HM790, China) containing the pre-cultures (*S. cerevisiae* Y10 and *T. delbrueckii* Y22 monoculture and co-culture) until a concentration of approximately 8.0 Log CFU/g flour was reached. Mixed yeast fermentation trials were simultaneously inoculated with *S. cerevisiae* Y10 and *T. delbrueckii* Y22 cultures at 5:1, 1:1 and 1:5 ratios. Dough fermentation experiments were conducted at 30 °C and 85% relative humidity in a controlled fermentation cabinet (Bilon HWS180, China). Samples were collected over a period of 24 h and processed immediately for yeast growth. For carbohydrate and organic acid analysis, the dough samples were dried by lyophilising immediately after sampling. The samples were obtained from three different points of dough (approximately 2 cm from the surface of dough). The 0 min fermentation time point is the measurement point immediately after dough mixing and before the start of fermentation.

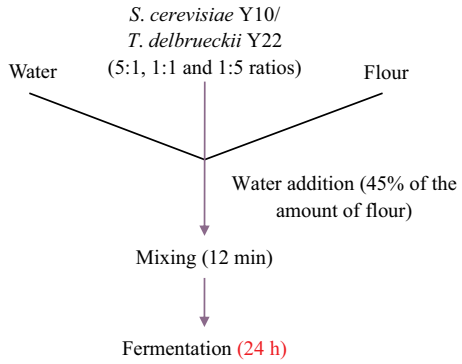

Fig. 1. Schematic representation of the dough preparation.

2.3. Cell population count of each species

The number of viable yeast cells of each species in the dough was determined by the traditional plate count method immediately after sampling. The samples were homogenised and diluted appropriately in 0.1% peptone water (Wang et al., 2018), and the yeast cells were enumerated by using Wallerstein Laboratory Nutrient (WL) agar (Pallmann et al., 2016) containing 30 μg/mL chloramphenicol. Plates were incubated at 28 °C for 3–5 days before counting. The cell population of each species in the dough with co-culture was estimated based on the distinct colony colour of *S. cerevisiae* Y10 and *T. delbrueckii* Y22 (Supplementary Fig. S1). The species were confirmed by restriction fragment length polymorphism (RFLP) analysis of the 5.8S rRNA gene and two ribosomal internal transcribed spacers with the restriction endonucleases *Hae*III (Esteve-Zarzoso et al., 1999) (Supplementary Fig. S2). The analyses were performed in three biological replicates.

2.4. Leavening ability

The leavening ability of the yeast was evaluated by measuring dough leavening and CO_2 production. Dough leavening was determined as described previously but with modifications (Perricone et al., 2014; Wahyono et al., 2016). Dough containing 100 g of flour, 45 mL of water and 8.0 Log CFU/g flour of yeast cells (*S. cerevisiae* Y10/*T. delbrueckii* Y22 at a ratio of 1:1) was mixed thoroughly. After mixing, 100 g of dough was placed in a 250 mL graduated cylinder. All samples were incubated at 30 °C for 3 h, and the increase in dough volume was measured every 30 min. This procedure was performed in triplicate for each sample. Rheofermentometer F3 (Chopin, Villeneuve-La-Garenne Cedex, France) was employed to estimate the total CO_2 formation during fermentation. Immediately after mixing, 315 g of the dough sample was placed in a cylindrical fermentation basket with an additional 2.0 kg weight disc imposed on top. The proofing chamber was closed tightly, and the dough was fermented at 30 °C for 180 min in accordance with the manufacturer's instructions. CO_2 volume was repeatedly measured at each 90 s interval and the total CO_2 volume produced at each 90 s interval is expressed as hydrostatic pressure in mm. This process was performed in two biological replicates, and the results were presented as arithmetic average.

2.5. Carbohydrate and organic acid analysis

Soluble carbohydrate and organic acid samples were prepared as described previously (Jayaram et al., 2013). Before analysis, Carrez precipitation was performed to eliminate proteins, fats and long-chained sugars from the dough samples (Wang et al., 2018). Reducing sugar was analysed by the 3,5-dinitrosalicylic acid method using a maltose standard curve (Miller, 1959). Glucose and maltose contents were determined with commercially prepared K-SUFRG and K-MASUG kits (Megazyme, Ireland), respectively (Katina et al., 2009). Organic acid levels were measured using an anion chromatography system (Dionex ICS-3000, USA) (Safa and Soucy, 2014; Wojtczak et al., 2013). Separation was conducted using an Ion Pac AS11 4 × 250 mm column and a ASRS-300 4 mm conductivity suppressor. Sodium hydroxide (1 mM) was employed as eluent with a flow rate of 0.6 mL/min. Amino

acid analysis was performed with an amino acid analyser (Sykam S433D, Germany) as described previously (Liu et al., 2017). All analyses were performed in two biological replicates with two technical replicates each.

2.6. Statistical analysis

Data were subjected to ANOVA by Tukey's test ($p < 0.05$) using the SPSS software (SPSS 19.0, SPSS Inc., Chicago, USA) and were reported as mean and standard deviation.

3. Results and discussion

3.1. Yeast growth

The evolutions of *T. delbrueckii* Y22 and *S. cerevisiae* Y10 during dough fermentation are shown in Fig. 2. The maximum cell populations of *S. cerevisiae* Y10 in the co-culture reached similar levels as those of the monoculture after 24 h, even at a *S. cerevisiae* Y10/*T. delbrueckii* Y22 ratio of 1:5 ($p > 0.05$). In *T. delbrueckii* Y22 monoculture, the cell population gradually increased to approximately 8.5 Log CFU/g wet dough. However, the *T. delbrueckii* Y22 populations in the co-cultures fluctuated and slightly changed throughout the fermentation. Significant differences in the *T. delbrueckii* Y22 populations were observed between the momoculture and co-culture after 24 h ($p < 0.05$). The results indicated that the growth of *S. cerevisiae* Y10 was not limited by *T. delbrueckii* Y22, regardless of the combination. On the contrary, *S. cerevisiae* Y10 suppressed the growth but did not cause the death of *T. delbrueckii* Y22.

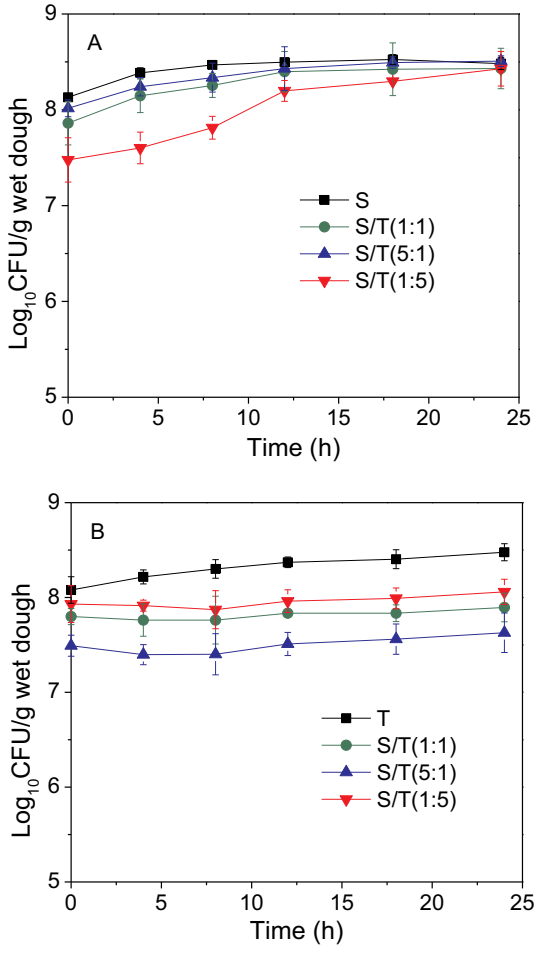

Fig. 2. Growth of *S. cerevisiae* Y10 (A) and *T. delbrueckii* Y22 (B) during dough fermentation with *S. cerevisiae* Y10 and *T. delbrueckii* Y22 monoculture and co-culture.

Although *T. delbrueckii* Y22 is not a strongly competitive yeast, it could survive at a dough environment with high number of *Saccharomyces* species, which is consistent with previous findings (Chen and Liu, 2016). *S. cerevisiae* slightly affected the population size of *T. delbrueckii* Td com (Wang et al., 2016). Sometimes, non-*Saccharomyces* and *Saccharomyces* cell populations can reach similar levels towards the end of fermentation (Bely et al., 2008). The viability of *T. delbrueckii* was also reduced in the *S. cerevisiae* and *T. delbrueckii* co-culture for long incubation times (Tronchoni et al., 2017). The peptides secreted by *S. cerevisiae* CCMI 885 can inhibit the growth of *T. delbrueckii* (Albergaria et al., 2010). The antagonistic effects may also be mediated by a cell-to-cell contact mechanism at high *S. cerevisiae* cell densities, and *T. delbrueckii* shows a decreased ability to compete for space in mixed cultures with *S. cerevisiae* (Nissen and Arneborg, 2003; Tronchoni et al., 2017). This early death is especially marked at high inoculum ratio between *S. cerevisiae* and *T. delbrueckii* during liquid fermentations (Toh et al., 2018). However, these interactions may not be particularly notable in the present study due to the rigid gel behaviour of dough, which is more solid-like than liquid-like. The contradictions might also be due to the substantial differences in the following fermentation conditions: sugar, nitrogen, oxygen and inoculum concentrations and incubation times and species (Bely et al., 2008; Chen and Liu, 2016; Wang et al., 2016).

T. delbrueckii Y22 and *S. cerevisiae* Y10 are clearly associated, and their ratio must be considered when designing a starter culture. Improved qualities of the steamed bread, such as high specific volume, whiteness and sensory score, were obtained when the yeast ratio of 1:1 was used (Supplementary Table S1). Therefore, this ratio was selected for further experiments.

3.2. Leavening ability

The leavening ability of *S. cerevisiae* Y10 and *T. delbrueckii* Y22 co-culture was investigated and compared with that of the monocultures (Fig. 3). For all treatments, the dough samples were greatly leavened in the first 1 h (Fig. 3A). The dough samples with *S. cerevisiae* Y10 monocultures initially reached the highest height at 2 h and then decreased gradually. For the dough incubated with *T. delbrueckii* Y22 monoculture, the dough height was low throughout the process. The dough with the *S. cerevisiae* Y10 and *T. delbrueckii* Y22 co-culture reached the maximum dough height after 3 h and then remained constant. *S. cerevisiae* Y10 monoculture showed higher leavening capacity than the co-culture used in the first 2 h ($p < 0.05$) (Fig. 3A). A decrease in dough volume was observed after 3 h. The volumes of dough inoculated with *S. cerevisiae* Y10 monoculture and *S. cerevisiae* Y10 and *T. delbrueckii* Y22 co-culture after 2.5 h of fermentation were not significantly different ($p > 0.05$). Therefore, incorporating *T. delbrueckii* Y22 with *S. cerevisiae* Y10 produced a co-culture with satisfactory dough leavening ability. This finding is in agreement with previous results showing that the co-culture of *S. cerevisiae*, *T. delbrueckii* JK08 and *P. anomala* JK04 in a dough system delivers favourable effects, such as improved bread quality and leavening ability for a long time (Wahyono et al., 2016). The low performance of *T. delbrueckii* compared with that of *S. cerevisiae* was also found in previous studies (Bely et al., 2008; Wahyono et al., 2016). On the contrary, high leavening abilities are exhibited by *T. delbrueckii* strains IGC5321 and IGC5323 under hyperosmotic and freeze–thaw stress (Hernandez-Lopez et al., 2003). These differences indicate that the dough leavening ability of *T. delbrueckii* is strain specific and is affected by substrates and environmental stress.

The CO_2 production profiles during the dough fermentation with monocultures and co-culture were also monitored. The CO_2 release of the monocultures of *S. cerevisiae* Y10 and *T. delbrueckii* Y22 at each 90 s interval increased rapidly in the first 1 and 0.5 h, respectively, and then

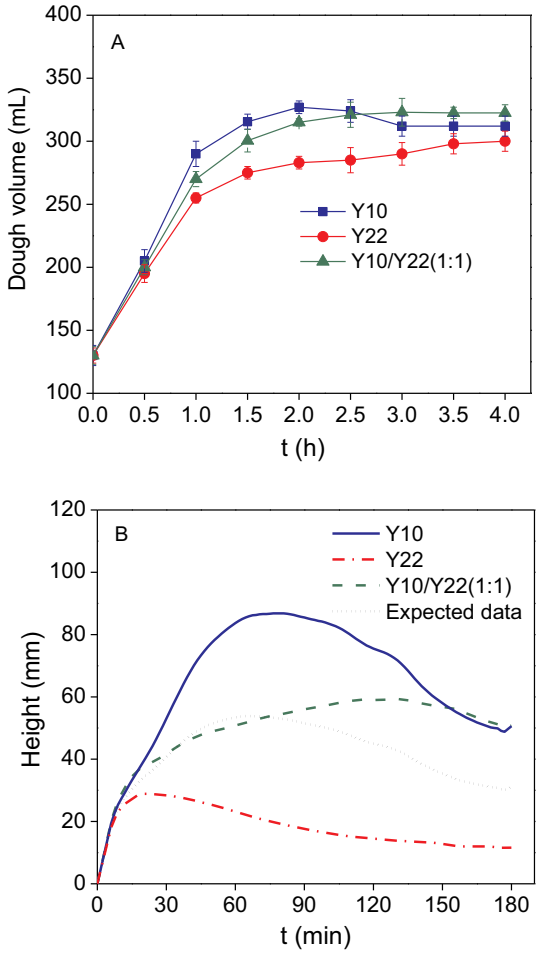

Fig. 3. Volume increase (A) and CO_2 release curves (B) of doughs inoculated with *S. cerevisiae* Y10 and *T. delbrueckii* Y22 monoculture and co-culture. Expected data line represents the arithmetic average of Y10 and Y22 monoculture.

decreased rapidly. The co-culture exhibited a rapid increase in CO_2 release at each 90 s interval in the first 0.5 h, which then remained relatively constant. The average CO_2 production of the monocultures of *S. cerevisiae* Y10 and *T. delbrueckii* Y22 was calculated (Fig. 3B). In the absence of interactions, the sum of half of the CO_2 production of the *S. cerevisiae* Y10 plus the half of the CO_2 production of the *T. delbrueckii* Y22 is theoretically equal to the CO_2 production in the co-culture. The expected and actual values of the total CO_2 produced by the dough incubated with co-culture for up to approximately 80 min were not significantly different. A gradually growing divergence was found between the two values, with the actual data always above the theoretical average of the monocultures. During this stage, the yeast numbers were similar for the monocultures and co-culture (Fig. 3), indicating the stimulation of CO_2 production and yeast metabolism during the dough fermentation.

The physiological interactions between *S. cerevisiae* and non-*Saccharomyces* are generally negative (Albergaria et al., 2010; Wang et al., 2016). The stimulated metabolic activities of *S. cerevisiae* and *T. delbrueckii* after short contact times (2 – 12 h) have been recently observed (Tronchoni et al., 2017). The high CO_2 production rates in the co-cultures correlate with the transcriptional up-regulation of the glucose fermentation pathway genes from *S. cerevisiae* and *T. delbrueckii* (Tronchoni et al., 2017). These stimulatory effects that occur in relatively short incubation times correspond to the direct response of *S. cerevisiae* and *T. delbrueckii* to the presence of each other (Ciani et al., 2016; Tronchoni et al., 2017). Therefore, the stimulatory effect of the yeast strains described in this work may show practical implications in the design of mixed starter cultures to improve dough fermentation and product quality.

3.3. Carbohydrate profiles

To evaluate the carbohydrate utilisation in the dough, we detected the changes in the levels of reducing sugar. As shown in Fig. 4, reducing sugar utilisation was not significantly different among the *S. cerevisiae* Y10 monoculture, *T. delbrueckii* Y22 monoculture and co-culture. When *S. cerevisiae* Y10 monoculture was inoculated into the dough, the content of reducing sugar decreased rapidly in the first 4 h and then stabilised. On the contrary, the reducing sugar in the dough with monoculture of *T. delbrueckii* Y22 increased during the entire fermentation. The concentration of reducing sugar for co-culture remained relatively stable. The data confirm that *S. cerevisiae* can remarkably consume carbohydrates (Bell et al., 2001; Jayaram et al., 2013). The reducing sugar levels in the unyeasted dough largely increased (Fig. 4). Therefore, the increase in the reducing sugar during the dough fermentation by *T. delbrueckii* Y22 can be attributed to the slower rate for carbohydrate consumption rate than for starch and fructan hydrolysis (Paramithiotis et al., 2006; Randez-Gil et al., 2013; Struyf et al., 2017; Zhou et al., 2017). The relatively stable concentration of reducing sugar in the dough with mixed yeasts indicated a balance between starch hydrolysis and carbohydrate consumption.

Changes in the glucose and maltose concentrations in the dough samples were also evaluated (Table 1). The glucose and maltose levels increased significantly in the unyeasted dough ($p < 0.05$) and accounted for > 88% of the total reducing sugar at 12 h. These data confirmed that maltose and small amounts of glucose were the major carbon source for the yeast in dough (De Vuyst et al., 2016; Gobbetti et al., 1995; Higgins et al., 2001; Randez-Gil et al., 2013). For the dough incubated with *S. cerevisiae* Y10 monoculture, glucose and maltose contents were depleted, which was consistent with the results on reducing sugar (Fig. 4). In the dough with *T. delbrueckii* Y22 monoculture, glucose was almost depleted, whereas maltose concentration increased to as high as 9.01 mg/g dry dough after 12 h of incubation. Glucose over maltose metabolism is strictly controlled in *T. delbrueckii* (Alves-Araújo et al., 2007). When glucose was still detectable in the medium, *T. delbrueckii* Y22 did not consume maltose, thus causing its continuous accumulation. When *S. cerevisiae* Y10 and *T. delbrueckii* Y22 were used as a co-culture for dough fermentation, glucose was almost depleted after 12 h of incubation, whereas a relatively high maltose concentration was observed (5.93 mg/g dough). The co-culture is composed of half *S. cerevisiae* Y10 monoculture plus half *T. delbrueckii* Y22

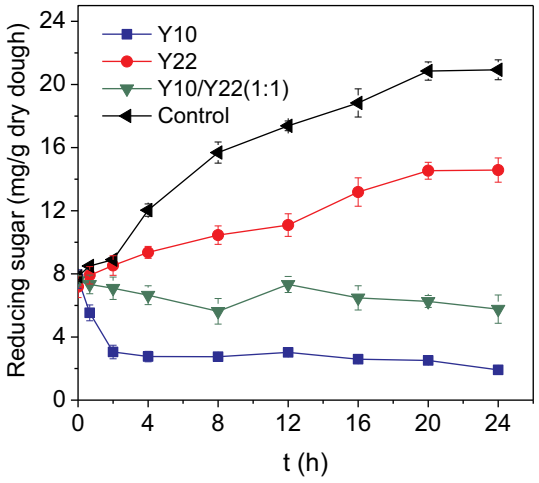

Fig. 4. Evolution of reducing sugar levels during dough fermentation with *S. cerevisiae* Y10 and *T. delbrueckii* Y22 monoculture and co-culture.

Table 1
Changes of glucose, maltose, lactic acid, acetic acid, succinic acid and EAA contents (mg/g dry dough) at the beginning and after 12 h dough fermentation.

	Time (h)	Glucose	Maltose	Lactic acid	Acetic acid	Succinic acid	EAA
Control[a]	0	0.54 ± 0.063	4.74 ± 0.47	0.18 ± 0.05	0.30 ± 0.03	3.10 ± 0.14	28.2 ± 1.3
	12	2.07 ± 0.105	13.38 ± 0.81	0.22 ± 0.06	0.34 ± 0.04	3.40 ± 0.11	28.5 ± 1.7
Y10	0	0.18 ± 0.027	3.75 ± 0.20	0.16 ± 0.07	0.34 ± 0.02	2.95 ± 0.12	28.7 ± 1.5
	12	0.02 ± 0.002	0.12 ± 0.10	0.16 ± 0.03	0.30 ± 0.03	4.01 ± 0.13	28.3 ± 1.2
Y22	0	0.16 ± 0.012	3.56 ± 0.30	0.21 ± 0.06	0.28 ± 0.08	3.10 ± 0.11	29.1 ± 0.2
	12	0.08 ± 0.007	9.01 ± 0.34	0.26 ± 0.09	0.30 ± 0.04	3.68 ± 0.15	29.5 ± 1.8
Y10/Y22 (1:1)	0	0.16 ± 0.031	3.54 ± 0.10	0.18 ± 0.04	0.38 ± 0.06	3.26 ± 0.28	29.3 ± 1.5
	12	0.03 ± 0.004	5.93 ± 0.24	0.19 ± 0.06	0.80 ± 0.08	6.64 ± 0.27	32.4 ± 0.4

[a] Control was the unyeasted dough.

monoculture. In theory, the sum of the halves of glucose and maltose utilisation by each kind of yeast is equal to the consumption of the sugars in the co-culture. In this case, maltose maybe partially consumed and is present at a relatively high concentration in the dough.

The changes in carbohydrate composition may cause an indirect response between *T. delbrueckii* and *S. cerevisiae* (Ciani et al., 2016; Tronchoni et al., 2017). The growth inhibition of *T. delbrueckii* Y22 (Fig. 2) in the presence of the *S. cerevisiae* Y10 may be attributed to an antagonism for carbohydrates (Nissen et al., 2004). The maltose transporter genes in *T. delbrueckii* have been repressed by the glucose, which restricts the external uptake of maltose (Alves-Araújo et al., 2007; Pacheco et al., 2012). *S. cerevisiae* plays a dominant role in the glycolysis of glucose and rapidly increases the consumption of sugars (Chen and Liu, 2016). In the current study, *T. delbrueckii* was less efficient than *S. cerevisiae* in utilising sugars in the co-culture. The leavening ability of dough was largely dependent on carbohydrate composition and yeast utilisation (Alves-Araújo et al., 2007; Zhou et al., 2017). Therefore, the carbon source utilisation behaviour showed the potential of the two yeasts for use as co-culture in dough fermentation to increase the persistence of CO_2 production and improve product quality.

3.4. Organic acids

The levels of succinic acid, lactic acid and acetic acid in the fermented dough were monitored (Table 1). Immediately after mixing, the initial concentration of each organic acid was similar in all dough samples ($p > 0.05$). The amounts of lactic acid and acetic acid remained nearly constant in the dough samples with *S. cerevisiae* Y10 and *T. delbrueckii* Y22 monocultures after 12 h dough fermentation. The *S. cerevisiae* Y10 and *T. delbrueckii* Y22 combination did not affect the lactic acid levels ($p > 0.05$), whereas the acetic acid increased ($p < 0.05$) after dough fermentation. Succinic acid content increased significantly in the fermented dough containing *S. cerevisiae* Y10 monoculture but only slightly in the dough with *T. delbrueckii* Y22. The amount of succinic acid increased significantly in the fermented dough with co-culture and was higher than that with *S. cerevisiae* Y10 monoculture ($p < 0.05$).

The lactic acid levels in the fermented dough were not significant changed, which disagreed with a previous report on the net consumption of lactic acid during dough fermentation (Jayaram et al., 2013). The lactic acid in the dough originates from the flour and conforms to the amounts of LAB detected (Ua-Arak et al., 2016). The flour used by Jayaram et al. (2013) was devoid of any LAB. However, in the present study, a large number of LAB (approximately 5 Log CFU/g wet dough) was found after 12 h. The stimulated metabolic activity of *S. cerevisiae* Y10 by *T. delbrueckii* Y22 may contribute to the net increase in succinic acid and acetic acid found in the dough with mixed yeasts because *S. cerevisiae* produces these acids (Jayaram et al., 2013).The anaerobic conditions of dough may favour the reduced succinic acid production (Raab and Lang, 2011). Spontaneously co-growing LAB produces a mixture of acetic acid and lactic acid (Gamel et al., 2015).

The organic acids formed by LAB and yeasts determine the dough acidification. Organic acids greatly affect the rheological properties of dough and the quality of the final product, such as the elasticity of gluten network, retention of dough gas and structure of the bread (Corsetti and Settanni, 2007; Ua-Arak et al., 2016; Zannini et al., 2009). In particular, the rheological properties depend greatly on final pH and the nature of the acid (Clarke et al., 2002; Ronda et al., 2014). Adding succinic acid may lead to the swelling and unfolding of the flour proteins (Jayaram et al., 2014). Appropriate pH and amounts of lactic acid and acetic acid can improve the product volume and gas retention capacity of the dough (Gobbetti, 1998; Rocha and Malcata, 2012). Biological acidification greatly increases loaf specific volume relative to the chemical acidification (Clarke et al., 2002). On the contrary, organic acids in the dough can increase the baker's yeast activity (increased CO_2 release), which subsequently results in good dough development (Moroni et al., 2012). However, extremely high acid concentration negatively affects the yeast growth and activity (Ua-Arak et al., 2017). Acetic acid and lactic acid have a synergistic effect on yeast activity as previously reported (Graves et al., 2007). In addition to succinic acid, lactic acid and acetic acid, yeasts produces several other metabolites, such as ethanol, carbon dioxide, hydrogen peroxide, glutathione, flavour compounds and enzymes (Jayaram et al., 2013), which can also strongly affect dough properties (Jayaram et al., 2014). In the current study, the difference in viscoelastic behaviour of the dough samples with *S. cerevisiae* Y10 and *T. delbrueckii* Y22 monocultures and co-culture increased after dough fermentation (Supplementary Fig. S3).

The contents of essential amino acids (EAAs) of the fermented dough were also determined and are shown in Table 1. *S. cerevisiae* Y10 and *T. delbrueckii* Y22 monocultures slightly changed the concentration of EAA after 12 h of dough fermentation ($p > 0.05$). When Y10 and Y22 were combined, the concentration of EAA markedly increased ($p < 0.05$). In particular, Lys, the first limiting amino acid of wheat flour (Wu, 2009), was significantly increased ($p < 0.05$) (data not shown). Dough fermentation positively affects the nutritional features (protein content and quality) of leavened flour goods due to the metabolic activity of fermenting microorganisms (Mohammed Nour et al., 2018; Rizzello et al., 2016; Valerio et al., 2017). Therefore, the increment of EAA in this study confirmed the stimulated metabolic activity of yeasts in the co-culture and may positively affect the kinetics of acidification (Di Cagno et al., 2003).

Dough fermentation by mixed yeasts apparently presents conditions that improve product quality, which may be due to the synergistic effects of all metabolites excreted by the yeast cells. Although the high population of yeasts is an advantage in this study, possible interference from native flour microbiota could not be eliminated because it may also be responsible for several beneficial properties of the dough and product (Corsetti and Settanni, 2007; Ua-Arak et al., 2016). Yeast metabolism is the main source of aromatic diversity in alcoholic beverages and bread (Birch et al., 2013; De Vuyst et al., 2016). Thus, further studies must investigate synergism for aromatic diversity production in a co-culture.

In conclusion, the changes in sugar utilisation pattern and gas and organic acid production in the dough revealed positive interactions between the strains of *T. delbrueckii* Y22 and *S. cerevisiae* Y10 during fermentation. The mutual responses for these two strains are not unique. The co-culture of *T. delbrueckii* and *S. cerevisiae* shows potential application in dough fermentation for steamed bread making. Studies based on different levels (e.g. gene transcription and proteins) are interesting topics for future research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.05.009.

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