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The state of water is associated with the viability and acidification capacity of *Lactobacilli* in frozen sourdough

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Abstract: The correlations between water state and the key factors affecting sourdough quality, including cell activity and acidification capacity of lactic acid bacteria (LAB), were established in this study. Results revealed that with the increase of frozen storage time, the cell density (CD), total titratable acidity (TTA), and organic acids content declined, whereas the pH value rose. Further, the freezable water content (FWC) and free water quantity (FWQ) decreased, but the total water loss rate (WLR) and immobilised water quantity (IWQ) increased. The CD showed a highly inversely correlation with WLR, and the pH value was strongly inversely correlated to the FWQ. The confocal laser scanning microscopy (CLSM) observed that the ice crystals had larger volumes during frozen storage. Our data, for the first time, disclosed that the total water content and the FWQ may play a crucial role in maintaining the viability and acidification capacity of LAB in frozen sourdough.

Keywords: lactic acid bacteria; cell density; titratable acidity; freezable water; water mobility; water state

Sourdough, a traditional leaven agent, has been used in the flour-made food industry for thousands of years worldwide. The types of productions on the basis of fermented sourdough are numerous, such as Chinese steamed bread, typical and traditional breads, and a range of Italian sweet products (Chavan and Chavan 2011; Zhao et al. 2019). Both spontaneous and starter culture-initiated sourdough fermentation processes are adopted, mainly by artisan bakeries and industrial bakery companies, respectively (Weckx et al. 2018). The sourdough is routinely prepared by water and flour with a consortium of microorganisms, including yeasts and lactic acid bacteria (LAB). Typically, the number of the prevalent LAB species in sourdoughs is higher than that of the prevalent yeast species (De Vuyst et al. 2016, 2017; Van Kerrebroeck et al. 2017). LAB are essentially responsible for proteolytic activity, volatile, antibacterial, and antifungal compounds formation (Coda et al. 2018; Sadeghi et al. 2018; Ripari et al. 2019). These characteristics enable sourdough to improve

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the flavour and the nutritional value of productions. Moreover, they are contributive to the prolongation of the storage life and the resistance to microbial spoilage (Corsetti and Settanni 2007; Gobbetti et al. 2014).

It is very difficult to control the stable preservation of traditional sourdough because its microbial ecosystem can be easily affected by environmental factors, for instance, the ingredients in use and the type of flour, the storage temperature, the number of refreshments made, the hygienic conditions of the processing environment and the operator (Minervini et al. 2014; Reale et al. 2019). To date, there are several ways to store traditional sourdough via refrigerated, frozen or dried strategies. Among these preserving technologies, frozen storage could partially maintain cell density (CD) and acidification capacity of LAB up to 90 days, while refrigeration and dried storage kept the good quality no more than 30 days for the storage (Santivarangkna et al. 2010; Lattanzi et al. 2014). Therefore, the frozen storage method may be an effective way to the long--term storage of the sourdough. However, freezing tends to cause injuries for the cell wall, membrane and ribonucleic acid, which leads to the loss of cell viability (Frédéric et al. 2003; Castro et al. 2010).

The freezing-derived cell injury by freezing resulted from the intracellular ice formation and the solution effects. With the rapid cooling rate, intracellular water forms an ice nucleus. The ice crystals give rise to mechanical damage to the cell membrane, leading to inner cell injury. On the other side, in case the cooling rate is slow, ice crystals will form in the extracellular solution first, resulting in the increase of electrolyte concentration of unfrozen fraction. The process may induce cellular dehydration by osmotic pressure. Cells hence can be potentially harmed along with the intensification of dehydration injured, and this is referred to as the 'solution effects' (Han and Bischof 2004). However, it is largely unknown how ice crystals or the water state influence the survival of LAB cells in the frozen sourdough.

The aim of this study is to establish correlations between the water parameters and the viability or the acidification capacity of LAB and further to find the key factor affecting the survival of LAB in the frozen sourdough. Therefore, CD of LAB, pH value, titratable acidity (TTA) and organic acids of sourdough were evaluated during frozen storage. Additionally, water loss rate (WLR), freezable water content (FWC), free water and immobilised water quantity (IWQ) were also determined by differential scanning calorimetry (DSC) and low field nuclear magnetic resonance (LF-NMR), respectively. The confocal laser scanning microscopy (CLSM) was used to observe the volume changes of ice crystals. Altogether, the data will cast light on the improvement of frozen sourdough quality by regulating the water state.

MATERIAL AND METHODS

Strain and growth conditions. *Lactobacillus plantarum* M616 was preserved in our laboratory at –80 °C (U535; Eppendorf, Germany) with 30% glycerol (v/v). As a starter for fermentation, M616 were routinely propagated at 37 °C for 24 h (MAXQ4000; Thermo, USA). When the exponential phase of growth was reached, cells were harvested by centrifugation at 5 000 rpm for 10 min (5804; Eppendorf, Germany), washed with 50 mM phosphate buffer, and re-suspended in an aliquot of the tap water used for sourdough preparation.

Sourdough preparation and freezing conditions. Wheat flour [protein (12.5 g 100 g^{-1}), fat (1.7 g 100 g^{-1}), carbohydrate (75 g 100 g^{-1}), ash (0.7 g 100 g^{-1})] was purchased from the local supermarket. Wheat flour (200 g) and cell suspension (100 mL) containing about 9.0 log colony-forming unit (CFU) mL^{-1} of LAB were used to prepare 300 g of dough. The dough was divided into aliquots of 10 g. Following that, the samples were packed in polyethylene bags (Q2538, Qujiebao, China) and fermented at 37°C for 24 h (MAXQ4000; Thermo, USA).

After fermentation, the samples were frozen in a trial freezer (DW-FW110 Zhongke Meiling; Cryogenics Corp., China) at –40 °C until the temperature in the centre of samples reached –18 °C. Then they were stored at –18 °C in a freezer (BCD-160TMPQ; Haier, China) for up to 8 weeks. These samples were taken out for an analysis on storage day 1, 7, 15, 21, 30, 45, and 60, respectively.

Preparation of common dough, dough fermented with lactic acid. The common dough was prepared with wheat flour (200 g) and water (100 mL). Parallelly, dough fermented with lactic acid was prepared with wheat flour (200 g) and lactic acid solution (5 g 100 mL^{-1}). The acidity of dough fermented with lactic acid was equal to sourdough after fermentation. Subsequent steps were the same as sourdough treatment.

Microbial counts. Frozen sourdoughs were thawed at 25 °C for 30 min [80% relative humidity (RH)] (LHS--150SC; Shanghai Yiheng, China). Sourdough (10 g) was homogenised with 90 mL of sterile sodium chloride (0.9%, w/v) solution. For the determination of LAB, serial dilutions were plated on De Man, Rogosa and Sharpe (MRS) agar supplemented with

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0.01% cycloheximide. After incubation at 37 °C for 48 h (LHS-150SC; Shanghai Yiheng, China), colonies were counted with the naked eye. The experiment was carried out on triplicate samples.

Determination pH, titratable acidity (TTA) and organic acids. Frozen sourdough was thawed as described above. Sample (10 g) was mixed with 90 mL sterile distilled water and homogenised for 2 min. The pH was recorded by a pH meter (EF28; Mettler-Toledo, Switzerland), and TTA was expressed as the volume of 0.1 N NaOH used to neutralise the solution to pH 8.3.

The suspension was filtered through 0.22 μm membrane filter (Jinteng, China). The analysis of lactic acid and acetic acid was performed by 1 200 HPLC system (Agilent, USA) coupled with a diode array detector. Samples were separated by a reverse-phase column (XDB C18, 150 mm × 4.6 mm, 5 μm; Agilent, USA) with a flow rate of 0.2 mL min^{-1}. An isocratic elution was performed with methanol (15%) and 0.02 mol L^{-1} monopotassium phosphate solution (85%). The detection wavelength was 210 nm. The sample in the volume of 10 μL was used for each experiment.

Water loss of frozen sourdough. Water loss of frozen sourdough was represented as the total mass change of dough. At certain time points of storage, frozen sourdough coating with the plastic bags were taken out of the freezer. The frozen samples were weighed (BSA223S; Sartorius, Germany). Subsequently, the samples were placed back into the freezer for storage until the next evaluation. The WLR was calculated with the following formula:

$$WLR(\%) = \frac{M_0 - M_d}{M_0} \times 100 \quad (1)$$

where: WLR – water loss rate; M_0 – mass of unfrozen sourdough; M_d – mass of sourdough at certain storage time points.

Measurement of freezable water content (FWC). The FWC was investigated by DSC (DSC 204F1; Netzsch, Germany). Sample (20 mg) was taken from the central portions of frozen sourdough and sealed hermetically into aluminium pans (JYL0040; Netzsch, Germany). All scans were administered by equilibrating at 25 °C for 3 min, cooling at 5 °C min^{-1} to –40 °C, holding at –40 °C for 10 min and heating at the rate of 5 °C min^{-1} up to 25 °C (204F1; Netzsch, Germany). An empty pan was used as a reference, and dry helium (25 mL min^{-1}) was used as the purge gas. Three replica samples were measured. Enthalpy of melting (ΔH) was calculated by software provided by the DSC manufacturer. The FWC of sourdough was calculated with the following formula (Ding et al. 2015):

$$FWC(\%) = \frac{\Delta H}{L \times W} \times 100 \quad (2)$$

where: FWC – freezable water content; L – known latent heat for the melting of ice (334 J g^{-1}); W – moisture content of the sample (g g^{-1}).

Low field nuclear magnetic resonance (LF-NMR). Proton relaxation measurements were performed by using a Proton (^1H) nuclear magnetic resonance (NMR) spectrometer (NMI20-015V-1; Shanghai Niumag Co. Ltd., China) to analyse the T_2 relaxation time. Approximately 3 g of dough were wrapped by polytetrafluoroethylene tape (Rike, China) to stop the water dry out and placed in a 15 mm diameter glass tube (Wilmad, USA). The duration between successive scans (TW), time of echo (TE), number of the scans (NS) and number of echoes (NECH) were 2 000 ms, 0.187 ms, 16 and 4 000, respectively. The data were fitted by T_2-fit program. The area under each peak was calculated by cumulative integration.

Confocal laser scanning microscopy (CLSM). Samples were cut into roughly 1 × 1 × 1 cm sections using a sharp blade, embedded in tissue freezing medium (Leica, Germany) and kept at –18 °C for 1 h. Using a cryostat microtome (CM1850; Leica, Germany), 20 μm thick sections were cut at –20 °C and fixed along with these components. Then starch and proteins were stained as green and red under room temperature, respectively (Lucas et al. 2018). Starch and proteins in samples were labelled with fluorescein isothiocyanate (FITC) (0.5 mg mL^{-1}) and rhodamine B (0.025 mg mL^{-1}), respectively, for 2 min, followed by washing three times with deionised water. Microscopic photos of frozen sections were imaged with a CLSM (LSM-710; Carl Zeiss, Germany) equipped with a computer. The excitation wavelengths for red and green labels were 488 nm and 561 nm, respectively.

Statistical analysis. Statistical analysis of the data was carried out by linear Pearson correlation coefficients (two-tailed), and one-way analysis of variance (ANOVA) using the SPSS 17.0 for Windows (SPSS Inc., USA), $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Cell density (CD) and acidification capacity of LAB in the frozen sourdough. The changes of CD and acidification capacity (pH, TTA and organic acids)

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Table 1. CD, pH, TTA, lactic acid and acetic acid level of sourdough during frozen storage (mean ± SD; $n \geq 3$)

Frozen storage time (days)	CD (log CFU g^{-1})	pH	TTA (mL)	Lactic acid (μg g^{-1})	Acetic acid (μg g^{-1})
0	7.5 ± 0.10[a]	3.70 ± 0.02[d]	14.9 ± 0.2[a]	13.76 ± 0.16[a]	5.58 ± 0.21[a]
1	7.0 ± 0.07[b]	3.74 ± 0.01[c]	13.9 ± 0.4[b]	11.68 ± 0.20[b]	5.34 ± 0.18[a]
7	6.9 ± 0.05[b]	3.77 ± 0.02[c]	13.3 ± 0.2[c]	9.42 ± 0.18[c]	4.99 ± 0.10[a]
15	6.8 ± 0.02[b]	3.85 ± 0.03[b]	11.9 ± 0.2[d]	7.92 ± 0.35[d]	4.75 ± 0.25[a]
21	6.7 ± 0.05[b]	3.89 ± 0.01[a]	11.6 ± 0.2[d]	6.41 ± 0.30[e]	4.28 ± 0.25[b]
30	6.5 ± 0.10[c]	3.90 ± 0.02[a]	11.4 ± 0.3[d]	4.90 ± 0.30[f]	3.80 ± 0.30[c]
45	6.2 ± 0.15[d]	3.93 ± 0.02[a]	10.9 ± 0.5[d]	4.51 ± 0.30[f]	3.30 ± 0.30[d]
60	6.0 ± 0.05[e]	3.96 ± 0.01[a]	9.8 ± 0.6[e]	4.20 ± 0.20[f]	2.80 ± 0.21[e]

[a–f]Data with different letters in the same column were significantly different ($P < 0.05$); CD – cell density; TTA – total titratable acidity; SD – standard deviation

of LAB during frozen storage of 60 days were evaluated in this study (Table 1). CD after day 1 frozen storage (7.0 ± 0.07 log CFU g^{-1}) was markedly lower than the unfrozen samples (7.5 ± 0.1 log CFU g^{-1}), suggesting that the survival of LAB cells was compromised through freezing. From this time onward, CD was shown to slightly decrease, ranging from 7.0 ± 0.07 log CFU g^{-1} to 6.0 ± 0.05 log CFU g^{-1} after 60 days in the storage.

Acidity is a crucial indicator for sourdough maturity which is associated with the organic acids produced by LAB (Mantzourani et al. 2018). In our data, the pH values of frozen sourdough were higher than those of the unfrozen sample. Similar trends were observed for the TTA, which were lower after frozen storage. The pH values showed a slight increase, whereas the TTA values dropped from day 1 frozen storage. To be exact, the pH values ascend from 3.74 ± 0.01 up to 3.96 ± 0.01; however, the TTA values descend from 13.93 ± 0.35 down to 9.80 ± 0.56. In agreement with the pH and TTA, the concentration of lactic and acetic acid decreased with the time increase of frozen storage. Generally, LAB remained acidification capacity after storage of 60 days since that pH and TTA values were 3.96 ± 0.01 and 9.80 ± 0.56, respectively.

During freezing, LAB cells were subjected to low water stress (Santivarangkna et al. 2010). Some compounds from flours, such as maltose, glutamate, and proline, may act as cryoprotectants to counteract the loss of water for the cell (Valdez and Diekmann 1993). These flours-related cryoprotectants played a crucial role in maintaining the viability and acidification capacity of LAB during storage at –18°C.

Water loss and freezable water changes in the frozen sourdough. Textural deterioration, such as drying and hardening, usually occurs in the frozen dough (Chen et al. 2013). In order to determine the water variation in sourdough, WLR and FWC were measured on storage day 1, 7, 15, 21, 30, 45, and 60, respectively. The results showed a continuous WLR increase for the frozen sourdough over the storage (Table 2). Accompanied by the phenomenon of water loss, the drying surface of frozen sourdough was observed.

DSC analysis rendered further insights into the states of water inside the sourdough. Compared with its fresh counterpart, frozen sourdough exhibited smaller melting enthalpy of ice, alongside the lowered freezable water (Table 2). With the time-prolonged storage, FWC decreased from 39.00% to 33.31%, a very low value after day 60 frozen storage. Similar evidences were obtained by Chen (2013), proving that the frozen bread exhibits remarkably lower FWC than the fresh counterpart (Chen et al. 2013). Such fact could be attributed to the recrystallisation of ice crystals, a process that large crystals develop on the melting of other small crystals due to temperature fluctuation or pressure variations during storage (Baier-Schenk et al. 2005; Petzold and Aguilera 2009). The growth of these ice crystals demands water to redistribute regionally or even globally. Therefore, the internal water of frozen sourdough tends to migrate externally, causing less freezable water retained inside the frozen sourdough.

Water mobility of frozen sourdough. Pulsed ^{1}H NMR was applied to investigate the water migration within frozen sourdough. The transverse relaxation time distribution curves are exhibited in Figure 1. To explain the differences of distribution curves between sourdough and dough, three sample types with different treatments were analysed, including the common

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Table 2. WLR, FWC and water distribution of sourdough during frozen storage (mean ± SD; $n \geq 3$)

Frozen storage time (days)	WLR (%)	Melting enthalpy of ice (J g^{-1})	FWC (%)	Peak area proportion of T_{21} (%)	Peak area proportion of T_{22} (%)	Peak area proportion of T_{23} (%)
0	0.00 ± 0.00^{h}	68.07 ± 0.90^{a}	40.76 ± 0.50^{a}	37.98 ± 0.10^{a}	58.43 ± 0.09^{c}	3.98 ± 0.02^{a}
1	0.48 ± 0.01^{g}	65.13 ± 0.50^{b}	39.00 ± 0.20^{b}	37.18 ± 0.09^{b}	58.61 ± 0.20^{c}	3.92 ± 0.05^{b}
7	0.54 ± 0.02^{f}	64.11 ± 0.50^{b}	38.39 ± 0.20^{c}	36.78 ± 0.20^{c}	58.66 ± 0.10^{c}	3.75 ± 0.03^{c}
15	0.70 ± 0.02^{e}	62.88 ± 0.30^{c}	37.65 ± 0.20^{d}	36.65 ± 0.10^{c}	59.61 ± 0.10^{b}	3.72 ± 0.03^{c}
21	0.78 ± 0.01^{d}	62.15 ± 0.50^{c}	37.22 ± 0.20^{d}	36.14 ± 0.10^{c}	59.34 ± 0.10^{b}	3.52 ± 0.02^{d}
30	0.95 ± 0.01^{c}	61.71 ± 0.50^{c}	36.95 ± 0.20^{d}	35.98 ± 0.10^{c}	59.98 ± 0.30^{a}	3.47 ± 0.01^{e}
45	1.29 ± 0.02^{b}	61.53 ± 0.40^{c}	36.84 ± 0.20^{d}	35.85 ± 0.09^{c}	60.00 ± 0.09^{a}	3.35 ± 0.02^{f}
60	1.40 ± 0.03^{a}	55.62 ± 0.90^{d}	33.31 ± 0.50^{e}	35.28 ± 0.10^{d}	60.25 ± 0.30^{a}	3.34 ± 0.00^{f}

$^{a–h}$Data with different letters in the same column were significantly different ($P < 0.05$); WLF – water loss rate; FWC – freezable water content; SD – standard deviation

dough, dough fermented with lactic acid, and sourdough fermented with *L. plantarum* M616. The curve of the common dough showed a typical T_2 relaxation time distribution. Three proton populations: T_{21} (0.01–3.05 ms), T_{22} (3.05–75 ms) and T_{23} (75–500 ms), represent bound water, immobilised water, and free water, respectively (Ding et al. 2015; Chen et al. 2017). Figure 1 showed the T_{21} peak of sourdough was shifted to earlier relaxation time compared to the common dough. Presumably, the sourdough fermented with LAB produced a variety of organic acids, such as lactic acids and acetic acids, most of which contain ^{1}H protons. These ^{1}H protons could also be detected as mobile protons by NMR. The T_{21} peak pattern of sourdough was affected thereafter. Moreover, dough fermented with lactic acids presented a similar T_{21} peak pattern with sourdough. This phenomenon also proved the shift of T_{21} peak of sourdough may be due to organic acids production. Hence the T_{21} peak areas of sourdough could not represent bound water content. The peak areas of T_{22} and T_{23} were discussed in the following part.

The peak area proportions corresponded to the quantity of different distributed water (Zhu et al. 2019). As shown in Table 2, the peak area proportion of T_{23} significantly decreased from 3.98 ± 0.02 on the day 0 to 3.34 ± 0.00 on the day 60, indicating that the free water quantity (FWQ) reduced during frozen storage ($P < 0.05$). FWQ reduction might be related to the formation of large ice crystals and water loss. Differently, the peak area proportions of T_{22} slightly increased af-

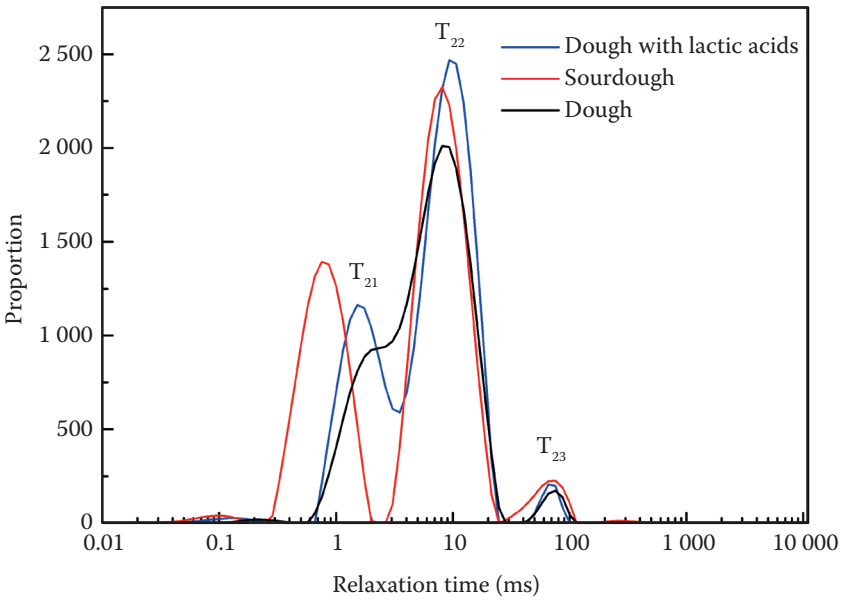

Figure 1. Nuclear magnetic resonance (NMR) spin-spin relaxation (T_2) curves of dough with three different treatments, the common dough, dough fermented with lactic acid, and sourdough fermented with *Lactobacillus plantarum* M616

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ter storage. It could be inferred that the frozen storage droved bound a water shift to less tightly bound water (Phimolsiripol et al. 2008; Yi et al. 2009). These results were consistent with the previously reported data that the decrease of T_{23} and the increase of T_{22} existed in frozen dough during 8 weeks of storage (Xin et al. 2018).

Correlation between viability of LAB and water parameters in frozen sourdough. The cell injury caused by freezing in microorganisms could be attributed to two factors, intracellular ice formation and solution effects (Han and Bischof 2004). The amount and size of ice crystals are associated with the content of freezable water in frozen dough (Xuan et al. 2017). With the formation of extracellular ice, cells are dehydrated, and the concentration of the intracellular solution increases caused by water migration. Therefore, investigation of the dynamic changes of the freezable water, water migration, and water loss in frozen sourdough could be useful to disclose the key factors affecting the viability of LAB.

The water state parameters were correlated with the index of cell viability. A linear Pearson correlation was used to describe relationships by means of correlation coefficients (R). The data were summarised in Table 3. CD showed a very highly inversely correlatation with WLR (P = 0.000, R = –0.996), indicating that WLR robustly affected CD, and the decrease of CD was along with WLR rising. The pH value was very highly significantly correlated to FWQ (P = 0.000, R = –0.966), suggesting that FWQ may play a crucial role in maintaining the acidified capacity of LAB. Correlations of TTA with water parameters were in accordance with pH values. In general, WLR, IWQ, and FWQ showed more strongly correlations with CD, pH, and TTA compared to FWC on the basis of the P and R value, suggestive of the fact that water content and distribution may be more important to maintain LAB activity than ice crystals.

Principal components analysis (PCA) for parameters of frozen sourdough. Principal components analysis (PCA) was used to extract the key factors which had effects on the quality of frozen sourdough. According to the requirements of total eigenvalues (≥ 1) and cumulative variance (≥ 85%), only component 1 was extracted, that total eigenvalues and cumulative variance were 6.503 and 92.895%, respectively (Table 4). Loading values reflect the influence degree of indexes

Table 3. Correlation coefficients (R) and level of significance (P) between WLR, FWC, IWQ, FWQ, CD, pH and TTA

Index of LAB activity	Correlation index	WLR	FWC	IWQ	FWQ
CD	*R*	–0.996	0.898	–0.890	0.927
	P	0.000	0.006	0.007	0.003
pH	*R*	0.923	–0.837	0.953	–0.966
	P	0.003	0.019	0.001	0.000
TTA	*R*	–0.935	0.905	–0.942	0.952
	P	0.002	0.005	0.001	0.001

LAB – lactic acid bacteria; WLR – water loss rate; FWC – freezable water content; IWQ – immobilised water quantity; FWQ – free water quantity; CD – cell density; TTA – total titratable acidity; correlated variables and coefficients are shown; $P \leq 0.05$ significant, $P \leq 0.01$ highly significant, $P \leq 0.001$ very highly significant

Table 4. Eigenvalue, contribution rate and cumulative contribution rate of the principal components

Component	Initial eigenvalues			Extraction sums of squared loadings		
	total eigenvalues	variance (%)	cumulative variance (%)	total eigenvalues	variance (%)	cumulative variance (%)
1	6.503	92.895	92.895	6.503	92.895	92.895
2	0.244	3.480	96.375	–	–	–
3	0.116	1.664	98.038	–	–	–
4	0.077	1.104	99.142	–	–	–
5	0.033	0.470	99.612	–	–	–
6	0.024	0.340	99.952	–	–	–
7	0.003	0.048	100.000	–	–	–

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acting on components. The greater absolute loading values are, the more influence degree between indexes and components are (Pang et al. 2021). Table 5 showed that the two biggest loading values were TTA (0.982) and WLR (–0.982), which indicated TTA and WLR were two key factors to evaluate the quality of frozen sourdough. Linear relation between indexes and component 1 were as follows: $F_1 = 0.150X_1 - 0.149X_2 + + 0.151X_3 - 0.151X_4 + 0.144X_5 - 0.143X_6 + 0.149X_7$.

Table 5. Eigenvectors and loading matrix of the principal components

	Component 1	
	eigenvectors	loading
CD	0.150	0.974
pH	–0.149	–0.970
TTA	0.151	0.982
WLR	–0.151	–0.982
FWC	0.144	0.937
IWQ	–0.143	–0.933
FWQ	0.149	0.968

CD – cell density; TTA – total titratable acidity; WLR – water loss rate; FWC – freezable water content; IWQ – immobilised water quantity; FWQ – free water quantity

Micro-structural observation of frozen sourdough. CLSM was used to observe the microstructure of sourdough during frozen storage. Some studies investigated wheat dough structure with rapid freezing by CLSM, of which protein networks were stained with rhodamine B. Results showed that a weakening of network structure was caused by freezing. Intermolecular bonds were ruptured due to the ice crystal formation of free water. The resulting gaps explained the increasing values for lacunarity (Lucas et al. 2018). In this study, frozen sourdough was composed of a mass of starch and proteins, tiny LAB cells, and amounts of ice crystals. Ice crystals could not be stained and would be washed out with deionised water. Before frozen storage of 15 days, black pores were small and distributed uniformly among starch particles and protein network. With ex-

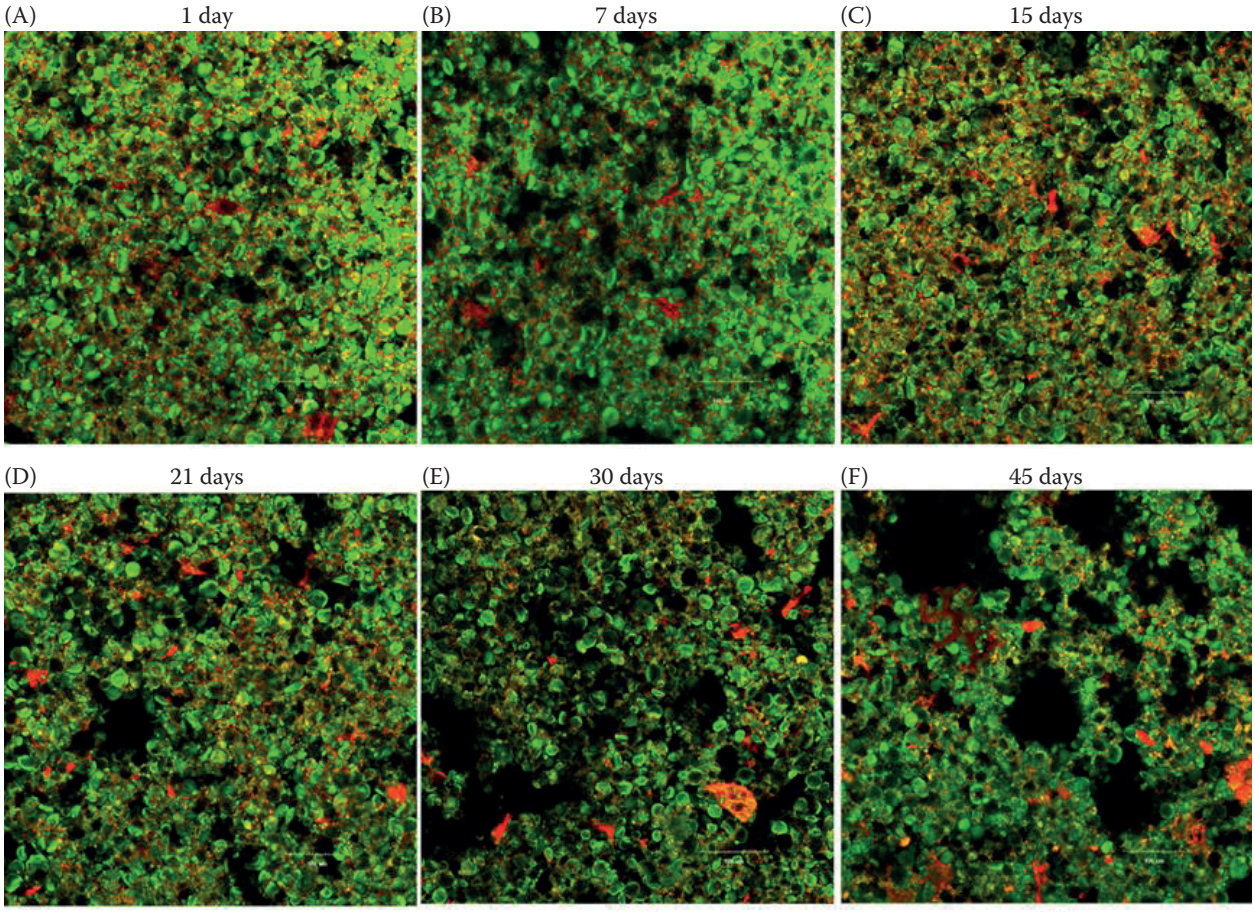

Figure 2. Confocal laser scanning microscopy (CLSM) micrographs of frozen sourdough stored at –18 °C for (A) 1 day, (B) 7 days, (C) 15 days, (D) 21 days, (E) 30 days, and (F) 45 days, respectively

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tended frozen storage, pores got larger and non-uniform. The larger dark pores were observed up to the day of 45 (Figure 2). The results indicated weakening protein networks in sourdough might be attributed to the aggregation of small ice crystals, accompanied by the formation of larger ice crystals during frozen storage. The results were in line with the data of FWC.

CONCLUSION

The viability and acidification capacity of LAB, as well as the state of water in frozen sourdough, were significantly changed during frozen storage of 60 days. With storage time increased from day 1 to day 60, CD and acidification capacity of LAB decreased. In addition, total WLR, FWC, FWQ decreased, and IWQ increased, suggesting that frozen storage greatly influenced water distribution in sourdough. The CLSM showed that large ice crystals gradually formed during frozen storage. Significant linear relations were observed among these parameters. CD and the pH value were very highly correlated to WLR and FWQ, respectively. In conclusion, total water content and free water might be the key factors affecting the viability and acidification capacity of LAB in frozen sourdough.

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