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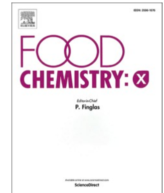

Investigation of *in situ* and *ex situ* mode of lactic acid bacteria incorporation and the effect on dough extensibility, bread texture and flavor quality during shelf-life

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ABSTRACT

Lactococcus lactis subsp. *diacetylactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lb. rhamnosus* were evaluated for their efficiencies in preserving bread texture and flavor during shelf-life. The investigated LABs exhibited efficiency during preliminary screening in expressing selected enzymes (protease, xylanase, α-amylase, laccase, and glucose oxidase) and producing exopolysaccharide (EPS). Dough samples were supplemented with either sourdough starters containing live LAB cells or LAB cell lysates. Prolonged fermentation further enhanced the protective advantage of sourdough starter incorporation. During the 5-day shelf-life period, *in situ* enrichment with *Lb. rhamnosus* led to a mere 12.5–35.4 % hardness change and 13.8–20.7 % overall texture change. Furthermore, sourdough bread with live LAB cell supplementation displayed a more diverse and intense flavor profile, with high concentration of bread key odorants maintained during shelf-life, including 2,3-butanedione, 2-acetyl-1-pyrroline, and 3-methylbutanal. Meanwhile, no significant improvement was found in bread enriched with LAB cell lysates during shelf-life.

1. Introduction

The reduction in bread freshness and deterioration of overall quality, along with the associating loss of consumer acceptance for the product over the storage period, has been a continuous source of concern for the baking industry, due to the substantial economic loss and waste generated. Voluminous research had therefore been conducted with the hope of tackling such issue (Melini & Melini, 2018; Alhendi & Choudhary, 2013). The previous focus on protecting bread products against microbial spoilage, has been progressively shifted towards addressing texture, flavor, the overall sensory quality changes, in bread products post-production (Di Monaco et al., 2015; Sun et al., 2020; Taglieri et al., 2021; Korcari et al., 2021). Texture modification characterized mainly as "staling", manifests through the firming and hardening of the bread crumb, while the crust softens concurrently (Curti, Bubici, Carini, Baroni, & Vittadini, 2011). Flavor changes, including the loss of desirable bread volatiles and increase of off-flavor compounds, can both impart adverse effect on bread flavor quality during shelf-life (Prost et al., 2020). Additionally, the desire of consumers in wanting "clean label" products free of synthetic preservatives also needs to be considered while trying to prolong the quality shelf-life of bakery products (Traynor, Martin, Ahmad, & Alonso, 2021; Vargas & Simsek, 2021). Therefore, strategies involving the incorporation of lactic acid bacteria (LAB) can be a competitive candidate, in providing comprehensive assurance against microbial, textural, and flavor changes. LAB's existing applications in different food products including bread, are due to their beneficial effects (De Vuyst et al., 2021; Bintsis, 2018; Wang et al., 2020). This study hypothesizes that *in situ* and *ex situ* LAB application can oppose the alteration and deterioration of both texture and flavor properties of bread, of both crust and crumb components during shelf-life.

The LAB strains selected for this study (*Lactococcus lactis* subsp. *diacetylactis* (RBL 37), *Lactobacillus delbrueckii* subsp. *bulgaricus* (RBL 52), and *Lactobacillus rhamnosus* (RBL 102)) demonstrated a mix of capabilities in expressing enzymes (protease, xylanase, α-amylase, laccase, and glucose oxidase), in producing exopolysaccharide (EPS), as well as in enhancing dough rheology and bread texture for freshly baked bread (Dong, Ronholm, Fliss, & Karboune, 2024). In the current study, these LAB strains are tested as a single strain or in dual combinations, through *in situ* (sourdough starter containing live LAB cells, designated as "SD") or *ex situ* (LAB cell lysates, designated as "MD") mode of LAB incorporation. By differentiating between single strain and two strains, the

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objective is to investigate the potential synergism on bread quality by using two LAB strains from different species. The *in situ* and *ex situ* way of incorporation enable evaluation of LAB cell lysate actions with or without their innate microbial characteristics. The duration of fermentation time is introduced as an additional variable for the *in situ* SD experiment. Throughout the fermentation period, the strength and quality attributes of all SD and MD dough samples are monitored, by quantifying dough extensibility parameters. After baking and during shelf-life, the texture, flavor and physical qualities of all bread samples are evaluated at predetermined intervals.

2. Materials and methods

2.1. Selection and growth condition of lactic acid bacteria

The strains used for the sourdough (SD) and LAB cell-lysate-enriched (MD) breadmaking experiments were *Lc. lactis* subsp. *diacetylactis* (RBL 37), *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52), and *Lb. rhamnosus* (RBL 102)(Dong, Ronholm, Fliss, & Karboune, 2024). They were selected according to their enzyme activities and/or EPS production abilities (*Supplementary Table 1*). Before experimental use, the strains were propagated once on MRS agar (BD Difco™, Thermo Fisher Scientific Inc., Massachusetts, USA) and further inoculated in MRS broth (BD Difco™, Thermo Fisher Scientific Inc., Massachusetts, USA) at 37 °C for 24 h under anaerobic conditions. Specifically, *Lb. rhamnosus* (RBL 102) as a single strain, and a combination of *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52), and *Lb. rhamnosus* (RBL 102) are evaluated in the SD setting; *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52) as a single strain, and a combination of *Lc. lactis* subsp. *diacetylactis* (RBL 37) and *Lb. rhamnosus* (RBL 102) are evaluated in the MD experiment.

2.2. Sourdough preparation and breadmaking

Colonies of each selected LAB strain were inoculated individually (inoculum level 1.0 %, *v*/v) into Erlenmeyer flasks containing 200 ml of MRS broth and incubated for 24 h at 37 °C under gentle agitation at 150 rpm. The preparation procedure of both yeast and LAB cell suspensions after incubation, containing 2×10^{10} cfu/ml for LAB cells and 10^7 cfu/ml for yeast cells, was consistent with the preceding experimental procedure (Dong et al., 2024). The cell culture was subsequently applied to prepare for the SD starter cultures. The yeast and LAB cell mixtures prepared were as follows: C-SD0: *S. cerevisiae*; C-SD1: *S. cerevisiae* and *Lb. rhamnosus* (RBL 102); C-SD2: *S. cerevisiae, Lb. delbrueckii* subsp. *bulgaricus* (RBL 52), and *Lb. rhamnosus* (RBL 102). C-SD0 was used as the control. The technical specifications of the unbleached wheat flour (Supérieure Flour, Les Moulins de Soulanges, Quebec, Canada) used for preparation of sourdough starter and dough samples are as follows: humidity: max 14.5 %; protein: 12.7 % ± 0.5; ash: 0.58 % ± 0.02; water absorption: 61.0 % ± 2.0; falling number: 290–330. No analysis on bacterial presence in the tap water used for sourdough starter preparation and breadmaking had been carried out for this study.

A three-stage technique was used for the preparation of sourdough following a modified version of the method described by Paramithiotis, Gioulatos, Tsakalidou, & Kalantzopoulos (2006). Dough 1 (d1) was prepared by thoroughly mixing 75 ml of the yeast and LAB cell suspension, with 300 g of unbleached wheat flour (Les Moulins de Soulanges, Quebec, Canada) and 225 ml tap water. After 24 h incubation at 25 °C, sourdough 1 (sd1) was formed. Dough 2 (d2) was formed by mixing 150 g of sd1 with 300 g of wheat flour, and 300 ml of tap water. Sourdough 2 (sd2) was formed after incubating d2 at 30 °C for 24 h. 150 g of sd2 was then added to 300 g of wheat flour and 300 ml of tap water in order to form dough 3 (d3), sourdough 3 (sd3) was formed after 24 h incubation at 30 °C. To make the final dough and for breadmaking, sd3 was used.

The final dough was made by mixing 765 g wheat flour, 135 g gluten, 180 g sd3, and 709.2 ml tap water for 10 min at speed 2 in the spiral mixer with dough hook attachment. 18 g of NaCl was added followed by another 5 min of mixing. The dough making procedure was carried out in duplicates. The resulting dough was then placed in a proofing cabinet for 4 h at 80 % relative humidity and 30 °C. 3 rounds of stretches and folds for the dough were performed every 30 min during the proofing process. The proofed doughs were divided into 260 g pieces and shaped. Half of the shaped doughs were baked in loaf pans right away at 220 °C for 20 min, with 5 s of steam injection at the beginning of the baking process. After baking, the bread loaves were allowed to cool to room temperature. Two loaves were subjected to assessment (Day 0), while the rest of the loaves were placed and packaged in common polyethylene bread bags under room temperature until the end of the targeted shelf-life, awaiting further assessment (Day 5). The other half of the shaped doughs were placed in cloth-lined loaf pans, then covered and let to rest overnight at 4 °C. Baking and shelf-life monitoring of the loaves after ~17 h of refrigeration were carried out under the same condition as previously described.

2.3. LAB cell-lysate-enriched dough preparation and breadmaking

Colonies of each selected LAB strain were inoculated individually (inoculum level 1.0 %, *v*/v) into Erlenmeyer flasks containing 1800 ml of MRS broth, and incubated for 24 h at 37 °C under gentle agitation at 150 rpm. For the cultivation of *Lc. lactis* subsp. *diacetylactis* (RBL 37) exclusively, the LAB cells were grown in modified MRS broth supplemented with 20 % sucrose to stimulate exopolysaccharide (EPS) production. The preparation procedure of LAB cell suspension and subsequent cell lysis, was the same as described in Dong et al. (2024). The LAB cell lysates prepared were designated as follows: C-MD1: *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52); C-MD2: *S. cerevisiae*, *Lc. lactis* subsp. *diacetylactis* (RBL 37), and *Lb. rhamnosus* (RBL 102). For C-MD2, the total amount of LAB cells and subsequent cel lysates obtained were estimated to be double the amount of C-MD1. The technical specifications of the unbleached wheat flour used for preparation of MD dough samples are as previously described in *Section 2.2*. No analysis on bacteria presence in the tap water used for breadmaking had been carried out for this study.

843 g wheat flour, 150 g gluten, 37.5 ml of the LAB cell lysates, 18 g of instant dry yeast, 760.95 ml tap water, 3.57 g calcium propionate, 1.09 g sorbic acid, 10.9 g acetic acid, and 18 g of NaCl were measured and added together to make the dough by mixing for 15 min at speed 1 in the spiral mixer with dough hook attachment. For MD2 doughs, 75 ml of LAB cell lysates and 737.6 ml tap water were added instead of what had been previously described, in addition to exclusive enrichment with 1 % flour weight of Glucans-30 (Lallemand, Montreal, QC, Canada). The control dough samples contained no LAB cell lysates, and a total of 798.45 ml tap water was added. The subsequent proofing was the same as described in *Section 2.3*. All the doughs after dividing and shaping were baked right away under the same condition as described in *Section 2.2*. After baking, the bread loaves were allowed to cool to room temperature. Two loaves were subjected to assessment (Day 0), while the rest of the loaves were placed and packaged in common polyethylene bread bags at room temperature, awaiting further assessment at predetermined intervals throughout shelf-life (Day 6, Day 10, and Day 13).

2.4. Dough assessment

Change of dough extensibility throughout the proofing period was evaluated using a Brabender extensograph (Brabender OHG Duisburg, Germany). 150 g of dough samples were collected at each of the three points for SD dough samples: at the beginning of proofing (T0), at the end of proofing (T4), and after overnight refrigeration (T17). 150 g of dough samples were collected at each of the three points for MD dough samples: at the beginning of proofing (T0), and at the end of proofing (T4). Each dough piece was stretched by the extensograph by a hook until rupture.

2.5. Bread assessment post-production and during shelf-life

The different aspects of bread assessment as described below were carried out repeatedly on Day 0 for each batch of the SD and MD bread samples, as well as on Day 5 for the SD bread samples, and on Day 6, Day 10, and Day 13 for the MD bread samples.

2.5.1. Bread physical assessment

Bread weight was measured using a digital scale. Bread loaf volume was determined following a modified rapeseed displacement method described by Approved Method 10–05.01 (AACC, 2000), where pearled barley was used instead of rapeseed. The bread was placed in a container of known volume. The container was then filled to the brim with pearled barley and the weight of the pearled barley was measured. The volume (cm^3) and bulk density (g/cm^3) of bread were calculated using the bulk density of pearled barley at 0.83 g/cm^3 as reported by Felizardo and Freire (2018).

2.5.2. Bread flavor analysis

0.5 g or 1 g of bread crumb and crust samples were collected separately from each loaf and placed in 20 ml headspace vials (Sigma-Aldrich, St Louis, USA), that were then hermetically sealed using a crimper. The samples were prepared in duplicates and stored at -80 °C prior to analysis by gas chromatography–mass spectrometry (GC–MS). The headspace samples were injected into GC column (30 m × 0.25 μm × 250 μm). The temperature of the transfer line was set to 120 °C using helium (99.99 % purity) as the carrier gas. The programmed temperature increases were as follows: 4 min at 35 °C, then the temperature was increased at a rate of 10 °C/min to 240 °C, and was maintained at 240 °C for 2 min. The total time for this process was 26.50 min. The mass range was scanned between 35 and 400 *m/z* EI+. Flavor compounds were identified by matching their retention times and mass spectra with those available in NIST database, and further confirmed using their profile with those of examined standards. Octane, decane, acetic acid, propanoic acid, ethyl butaric acid, 3-methylbutanoic acid, methyl pentanol, ethanol, 2-methyl-1-propanol, 2-methyl-1-butanol, hexanol, 3-methylbutanal, methional, (Z)-2-nonenal, (E,Z)-2,4-decadienal, (E,Z)-2,6-nonadienal, 2,3-butanedione, 1-octen-3-one, ethyl acetate, ethyl octanoate, furfural, and 2-acetyl-1-pyrroline were used as standards.

2.5.3. Bread texture analysis

2 cm thick slices of bread samples were prepared, and texture was measured from the center of the loaf slice and top of the loaf slice, for crumb and crust analysis respectively. Both components were analyzed using a TA-XT Plus Texture Analyzer (Stable Micro Systems Ltd., Godalming, UK) equipped with a 25 mm diameter cylinder probe under 30 % strain and 25 g of trigger force. Additional conditions for the TPA measurement were as follows: pre-test speed of 1.5 mm/s, test speed of 2 mm/s, post-test speed of 10 mm/s.

2.6. Statistical analysis

All statistical analyses were conducted using one-way ANOVA (Analysis of Variance) procedures with XLSTAT software (Addinsoft, Paris, France), to distinguish the responses of dough and bread samples made from different LAB strains. Differences were reported at a significance level of $\alpha = 0.05$. Correlation analysis was carried out to determine the Pearson correlation coefficients using the Python programming language (Version 3.8.3) supported by the statistics library SciPy (Version 1.8.0).

3. Results and discussion

3.1. Dough extensibility

The Brabender extensograph analysis was carried out on dough samples with either *in situ* or *ex situ* LAB cell supplementation, alongside yeast control dough samples under the same experimental conditions to monitor the dough behavior throughout the fermentation process.

3.1.1. Sourdough extensibility

The result is tabulated in Table 1(a). A dough quality pattern combining both good resistance and extensibility is considered strongly desirable (Komlenić et al., 2010). Both SD1 and SD2 samples show a similar evolution of maximum resistance (*R*), which measures the maximum amount of stress the dough can endure against an external stretching force, throughout the fermentation process. SD1 (*Lb. rhamnosus*) and SD2 (*Lb. delbrueckii* subsp. *bulgaricus* and *Lb. rhamnosus*) display almost identical behavior with slight softening of dough during the proofing stage of T0 to T4, followed by a sharp increase of *R* after overnight refrigeration recorded at T17. In contrast, SD0 sample experiences a sharper decrease of *R* to 776 BU at T4. The final R reaches 1638 BU for all three SD samples. Meanwhile, a similar extensibility (*E*) pattern, is observed among the three SD samples at the three critical points evaluated, with more distinct changes observed at T17. Extensibility (*E*), which represents the distance of stretching obtained before dough rupture, can provide insight for the gluten protein interactions within the dough sample (Hadnađev et al., 2011). The evolution of energy parameter (cm^2) can be found in Fig. 1(a) and is useful in positively relating to the amount of energy required to stretch the test dough piece to its rupture point. Only minor differentiation in energy can be observed among the three SD samples during the fermentation period from T0 to T4, while more prominent differences emerge after overnight refrigeration.

The overall dough extensional characteristics are further reflected through the ratio between maximum resistance and extensibility (*R/E*), demonstrated in Fig. 1(c). The *R/E* ratio is indirectly related to the degree of dough expansion during proofing and the subsequent baking stage (Hadnađev et al., 2011). The *R/E* ratio is maintained from beginning to end of the 4 h fermentation period for SD1 sample and experiences an increase in dough resistance after overnight refrigeration. Examination of the *R/E* ratio pattern for SD2 sample, shows a good balance between resistance and extensibility of the dough sample, and aligns with findings from sourdough literature (Komlenić et al., 2010). At T4, adequate extensibility is maintained while dough resistance progressively reduces. This reduction in dough resistance continues during overnight refrigeration and finally reaches 10.4 at T17, which is similar to the yeast control final *R/E* ratio of 9.3. In general, the dough extensibility evaluation reveals the notable influence of SD incorporation on dough properties. The effect is particularly significant with the prolonged fermentation time, evidenced by the distinctiveness among the three dough samples at T17, as visualized in Fig. 1.

Similar conclusions have been drawn in sourdough literature, where SD incorporation has been found to have a reducing effect on maximum resistance to extension (*R*), *R/E* ratio, and energy, especially notable in doughs with longer fermentation time. However, it is worth noting that the control dough sample in these studies are typically prepared without sourdough starter addition, rather than using sourdough starter prepared with yeast cells as control, as done in the present study. The softening effect of SD inclusion in dough samples is considered to be the combined effort of various factors, including the proteolytic system within LAB, the microbial action of the LAB itself, the acidification of the dough environment, which is enhanced by a long fermentation procedure (Komlenić et al., 2010). While protease is not considered to be the predominant enzyme present in LAB cells for SD1 and SD2 dough samples (*i.e. Lb. delbrueckii* subsp. *bulgaricus* and *Lb. rhamnosus*), based on prior screening of their enzyme expression abilities (*Supplementary Table 1*), the action of other enzymes of interest established experimentally in these two LAB strains (xylanase, α-amylase, laccase, and glucose oxidase), all have independent benefits on improving dough extensibility and overall workability (Kornbrust, Forman, & Matveeva, 2012; Dong, Ronholm, Fliss, & Karboune, 2024), in addition to protease

Table 1
Dough extensibility parameters[a]. (a): sourdough (SD) dough; (b): LAB cell-lysate-enriched (MD) dough.

		Energy (cm^2)	Resistance to Extension (BU)	Extensibility (mm)	Maximum (BU)	Ratio Number	Ratio Number Max
(a): sourdough (SD) dough							
T0	Yeast	120	1162	104	1171		11.2
		(6.36)[b]	(86.72)	(4.45)	(45.09)	(0.56)	(0.59)
	Lb. rhamnosus (RBL 102)	109	1027	101	1051	10.2	10.4
		(6.50)	(58.41)	(6.18)	(63.71)	(2.87)	(1.90)
	Lb. delbrueckii subsp. *bulgaricus* (RBL 52) + *Lb. rhamnosus* (RBL 102)	105	1125	90	1185	12.5	13.1
		(8.27)	(70.92)	(6.50)	(80.62)	(2.57)	(1.62)
T4	Yeast	77	698	114	776	6.1	6.8
		(3.73)	(52.87)	(6.08)	(38.00)	(0.46)	(0.51)
	Lb. rhamnosus (RBL 102)	90	797	94	1010	8.5	10.8
		(2.85)	(70.37)	(5.25)	(58.56)	(0.53)	(0.79)
	Lb. delbrueckii subsp. *bulgaricus* (RBL 52) + *Lb. rhamnosus* (RBL 102)	99	1003	89	1034	11.3	11.6
		(5.62)	(73.95)	(6.03)	(62.31)	(0.51)	(0.54)
T17	Yeast	416	1638	176	1638	9.3	9.3
		(19.73)	(0)	(8.86)	(0)	(0.29)	(0.29)
	Lb. rhamnosus (RBL 102)	301	1638	130	1638	12.6	12.6
		(15.42)	(0)	(9.53)	(0)	(1.14)	(1.14)
	Lb. delbrueckii subsp. *bulgaricus* (RBL 52) + *Lb. rhamnosus* (RBL 102)	395	1638	158	1638	10.4	10.4
		(17.63)	(0)	(10.66)	(0)	(1.02)	(1.02)
(b) LAB cell-lysate-enriched (MD) dough							
T0	Yeast	154	1638	92	1638	17.85	17.9
		(11.31)	(0)	(8.49)	(0)	(1.63)	(1.70)
	Lb. delbrueckii subsp. *bulgaricus* (RBL 52)	182.5	1638	107.5	1638	15.55	15.55
		(12.02)	(0)	(19.09)	(0)	(2.76)	(2.76)
	Lc. lactis subsp. *diacetylactis* (RBL 37) + *Lb. rhamnosus* (RBL 102)	176	1638	87.5	1638	18.65	19.05
		(5.66)	(0)	(3.54)	(0)	(0.78)	(1.20)
T4	Yeast	85.5	869.5	86	989.5	10.1	11.45
		(0.71)	(45.96)	(5.66)	(111.02)	(1.13)	(0.49)
	Lb. delbrueckii subsp. *bulgaricus* (RBL 52)	88.5	851.5	97	901.5	8.8	9.3
		(4.95)	(88.39)	(0)	(54.45)	(0.99)	(0.57)
	Lc. lactis subsp. *diacetylactis* (RBL 37) + *Lb. rhamnosus* (RBL 102)	101.5	946	74	1441	12.75	19.6
		(3.54)	(100.41)	(4.24)	(278.60)	(0.64)	(16.26)

[a] T0: dough extensibility at the beginning of fermentation; T4: dough extensibility after 4 h of fermentation; T17: dough extensibility after 4 h of fermentation and overnight refrigeration.
[b] The corresponding standard deviations of the values in the row above are reported in brackets.

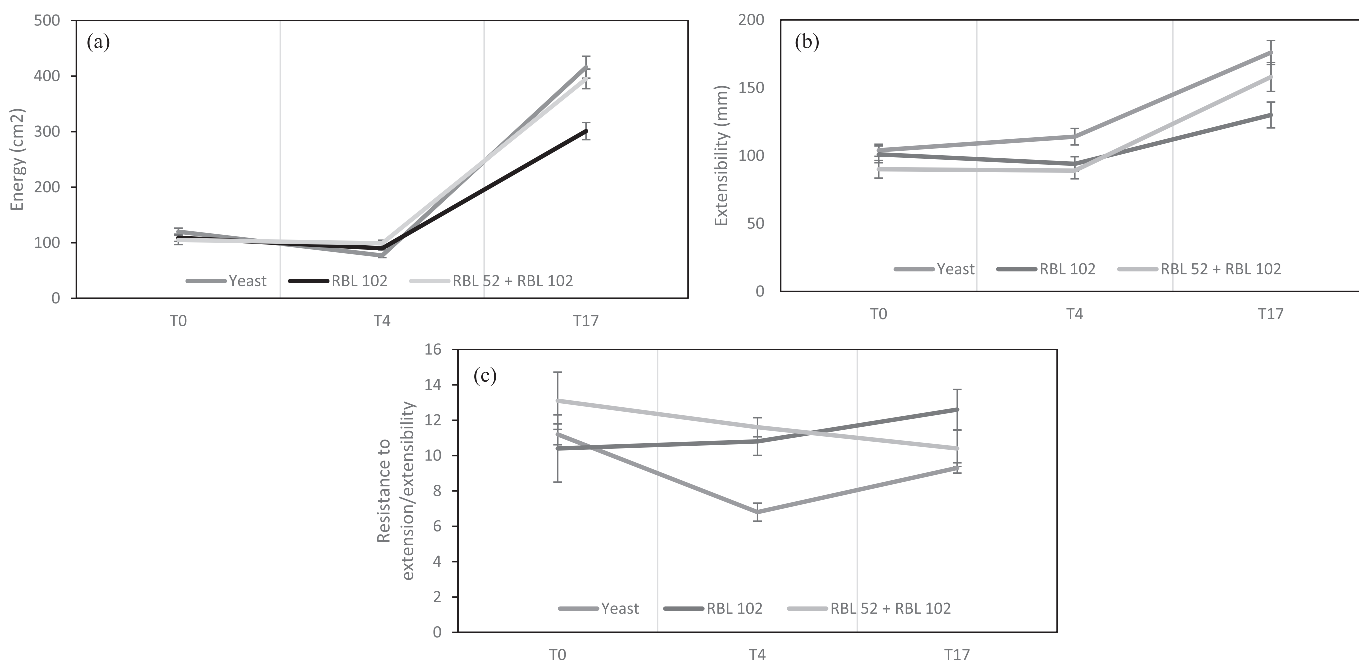

Fig. 1. Evolution of sourdough (SD) dough extensibility[a,b]. (a): evolution of energy; (b): evolution of extensibility; (c): evolution of resistance to extension/extensibility ratio.
[a] T0: dough extensibility at the beginning of fermentation; T4: dough extensibility after 4 h of fermentation; T17: dough extensibility after 4 h of fermentation and overnight refrigeration.
[b] RBL 102 refers to "*Lb. rhamnosus* (RBL 102)"; RBL 52 + RBL 102 refers to "*Lb. delbrueckii* subsp. *bulgaricus* (RBL 52) + *Lb. rhamnosus* (RBL 102)".

and other cereal enzymes naturally present in the flour (Kieliszek et al., 2021; Komlenić et al., 2010). This potential synergistic action is postulated and confirmed by the dough extensibility information previously discussed, that made an optimal environment and condition possible, facilitated by the presence of LAB cells.

3.1.2. LAB cell-lysate-enriched dough extensibility

For MD dough samples, extensibility is evaluated at the beginning of proofing (T0), and at the end of proofing (T4). MD1 dough made with *Lb. delbrueckii* subsp. *bulgaricus*, exhibits the highest initial energy among the three MD samples at 182.5 cm^2, with final energy that is similarly low as MD0 dough at 88.5 cm^2. On the other hand, MD2 sample that is prepared with the co-inclusion of *Lc. lactis* subsp. *diacetylactis* and *Lb. rhamnosus*, has a more balanced energy profile, and maintains an adequate final energy at 101.5 cm^2. A balanced energy profile is considered to positively relate to high dough mechanical strength and extensibility (Nawrocka et al., 2016). In terms of maximum resistance to extension (*R*), all three MD samples exhibit the same value prior to proofing, and subsequently, decrease to various degrees. Among the MD samples, MD2 experiences the smallest reduction in *R* with a final value of 1441 BU, as compared to 989.5 BU and 901.5 BU for the MD0 and MD1 sample, respectively. The addition of an external polysaccharide source in the form of yeast cell wall β-glucan in MD2 dough sample, combined with twice the amount of LAB cell lysates added during preparation as compared to MD0 and MD1, could provide insights into the noteworthy extensibility of MD2. The overall dough extensibility property, numerically represented by *R/E* ratio, follows a similar fashion as demonstrated in Fig. 2(c). MD2 dough maintains its profile adequately during the fermentation process, with an increase of *R/E* ratio from 19.05 at T0, to 19.6 at T4. Comparatively, MD1 sample and the yeast control MD0 sample both experience a decrease in *R/E* ratio. The MD experimental conditions allow for the observation of direct and independent effects of the LAB cell lysates (including enzymes and EPS) through *ex situ* incorporation, without interference of the LAB live cells. The dough quality and extensibility properties determined for the MD doughs are compared with literature conducted on dough samples supplemented with enzymes combinations, that are of interest to the present study. However, it is important to consider and highlight the limitation of such comparisons, as the supplementation levels of these enzymes added into dough vary greatly across different studies.

Insufficient information is available on studies of dough supplemented with all four enzymes of interest (*i.e.* xylanase, α-amylase, laccase, and glucose oxidase). Nevertheless, dough with the combinational addition of xylanase, glucose oxidase and/or laccase is commonly reported to exhibit increased *E*, decreased *R*, and overall decreased *R/E* ratio, as opposed to control doughs without enzyme supplementation (Konieczny et al., 2020). From a chemistry perspective, both glucose oxidase and laccase are suggested to improve protein-protein interactions, while presence of xylanase, assists in protein-water interactions (Konieczny et al., 2020). Therefore, when xylanase is used in conjunction with the cross-linkage promoting enzymes of glucose oxidase and laccase, it is postulated that xylanase can correct the effects of the loss in dough extensibility and gluten network hydration due to the arabinoxylan cross-linkages catalyzed by glucose oxidase and laccase. This is achieved through xylanase hydrolyzing arabinoxylan into small fragments, that releases water molecules previously sequestered by arabinoxylan, which promotes hydration and aggregation of the gluten and starch network (Dai & Tyl, 2021; Yang et al., 2021).

For the combinational effect of xylanase and α-amylase, impact on increase in *E* and decrease in *R* by applying both has been reported for Chinese steamed bread dough (Liu et al., 2017). The increase in *E* is suggested to be by xylanase redistributing moisture from pentosan to gluten (O'Shea, Kilcawley and Gallagher, 2016). Additionally, several other articles have also reported overall dough softening effects attributed to the action of both enzymes, in modifying and reducing the aggregation of starch and arabinoxylan fractions within the dough environment (Dai & Tyl, 2021; Kostyuchenko et al., 2021).

The two different ways of *ex situ* EPS incorporation, exclusively in MD2 dough, potentially add additional functional enhancement due to the hydrocolloid character of EPS structure. EPS can improve water retention capabilities of dough, while interact with major dough polymers, including gluten and starch, to strengthen the dough network, and thereby improves the overall dough extensibility and rheological behavior (Fadda et al., 2014; İspirli et al., 2020; Taglieri et al., 2021).

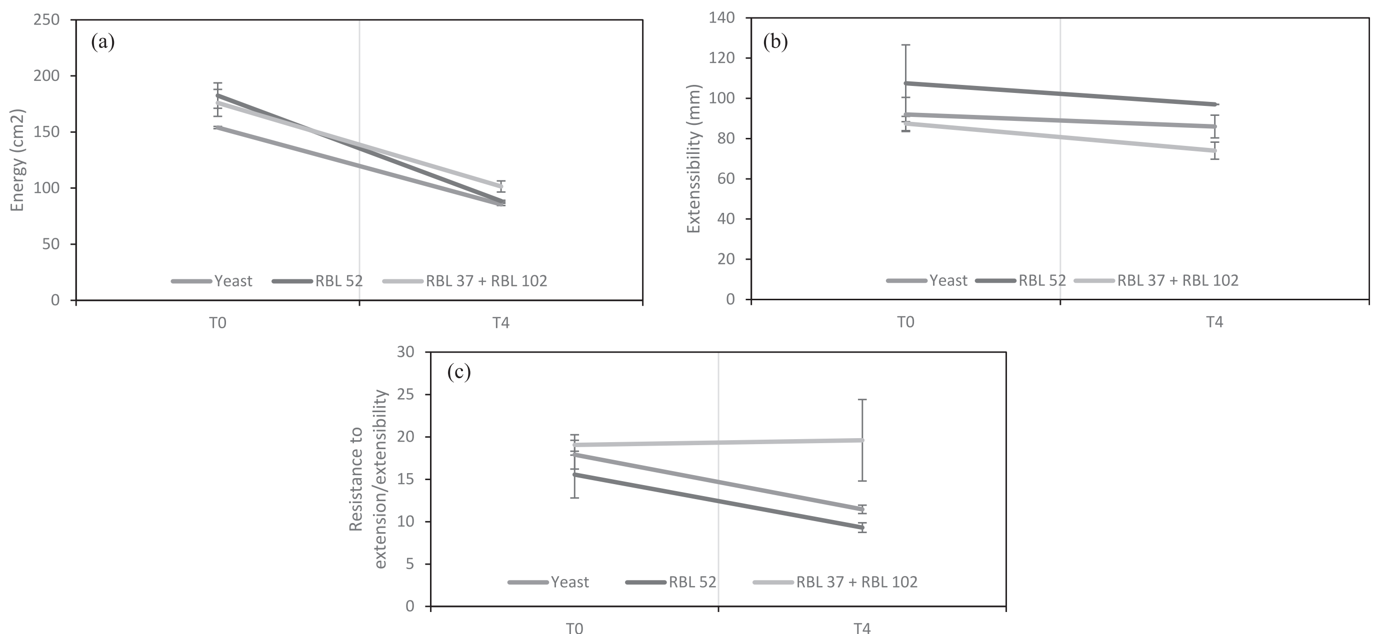

Fig. 2. Evolution of LAB cell-lysate-enriched (MD) dough extensibility[a,b]. (a): evolution of energy; (b): evolution of extensibility; (c): evolution of resistance to extension/extensibility ratio.

[a] T0: dough extensibility at the beginning of fermentation; T4: dough extensibility after 4 h of fermentation.

[b] RBL 52 refers to "*Lb. delbrueckii* subsp. *bulgaricus* (RBL 52)"; RBL 37 + RBL 102 refers to "*Lc. lactis* subsp. *diacetylactis* (RBL 37) + *Lb. rhamnosus* (RBL 102)".

Scarce information is available on the combined effects of both EPS and enzymes in dough samples, but previous studies have suggested that wheat starch pasting properties are modified when combining α-amylase with various hydrocolloids (for example: alginate, κ-carrageenan), which can contribute to viscosity and overall dough rheology (Palabiyik et al., 2016).

3.2. Evolution of bread texture during shelf-life

The texture evolution of bread samples of both crumb and crust components, is scrutinized within a pre-determined time frame, 5 days for SD, and 13 days for MD experiments. Comparing bread textural changes of samples with LAB based bio-ingredients incorporation to samples without, both post-production and during shelf-life, provides insights into the effectiveness and protective effect of such application, and thus, adequately validate the hypothesis of this study from a texture point of view. Additionally, the influence of fermentation duration that has long been established as an important factor on SD bread quality (Komlenić et al., 2010), is assessed in this study, by subjecting SD dough samples to either four hours of fermentation (4 h), or an additional overnight refrigeration (ON) period after four hours of fermentation, before being baked into bread loaves. It is noteworthy that all SD bread samples are without visible mould growth by the end of the 5-day shelf-life period. This observation aligns with typical microbial-free shelf-life of sourdough bread (Hayta & Hendek Ertop, 2018). No mould growth is observed for MD bread samples as well, due to the addition of external preservative agents (calcium propionate, sorbic acid, and acetic acid).

3.2.1. Sourdough bread texture during shelf-life

Bread loaves were prepared from respective sourdough starters containing C-SD0 (yeast), C-SD1 (yeast and *Lb. rhamnosus*), C-SD2 (yeast, *Lb. delbrueckii* subsp. *bulgaricus*, and *Lb. rhamnosus*) cell cultures. The texture behavior of the bread samples is visualized in Fig. 3.

For SD0 as shown in Fig. 3(a) and Fig. 3(b), the texture quality profile of the bread samples shows limited sensitivity to fermentation duration as confirmed by statistical analysis, both on Day 0 and Day 5, and for both crumb and crust. For bread crumb samples, cohesion, springiness, and chewiness levels differed significantly on Day 0 between 4 h and ON samples, while the difference between the two diminished on Day 5. For bread crust samples, the texture profile is distinctive between 4 h and ON samples, particularly on Day 5 among crust hardness, adhesiveness, resilience and gumminess. Overall, almost all texture attributes invariably display a high degree of change comparing Day 5 to Day 0 behavior, while chewiness level was maintained adequately during the 5-day shelf-life period for both crumb and crust.

The protective effect of LAB application on bread texture during shelf-life is undeniably demonstrated in SD1 bread, evidenced in Fig. 3 (c) and Fig. 3(d) by the minor texture change on Day 0 and Day 5, especially in ON bread crumb samples. Such advantage of an ON fermented bread could be attributed to not only the prolonged fermentation period, but also specifically to this study, the elevated initial crust hardness as indicated in Fig. 3(d). SD breads with longer fermentation typically have a thicker bread crust with a higher crust hardness (De Vuyst et al., 2021). The crust thus acts as a natural barrier to reduce and delay moisture and overall quality loss (Chen et al., 2021), which can help explain the well-maintained crumb profile represented in Fig. 3(c). Hardness, gumminess, and chewiness levels are lower in ON fermented bread crumb than in 4 h fermented bread crumb on Day 0, and remain to be so on Day 5. Meanwhile, bread crust behaves in an inverse fashion, with hardness, gumminess, and chewiness values higher in ON samples than in 4 h samples on Day 0, and comparatively lower on Day 5. The crust softening is to be expected in bakery products during storage, due to moisture uptake and water redistribution from the crumb towards the crust (Chiavaro et al., 2008; Ding et al., 2021).

SD2 bread samples display distinctive overall textural evolution patterns as compared to SD1 samples during shelf-life, according to Fig. 3(e) and Fig. 3(f). While SD2 bread crumb and crust have relatively low initial levels of hardness and chewiness, the bread crumb and crust

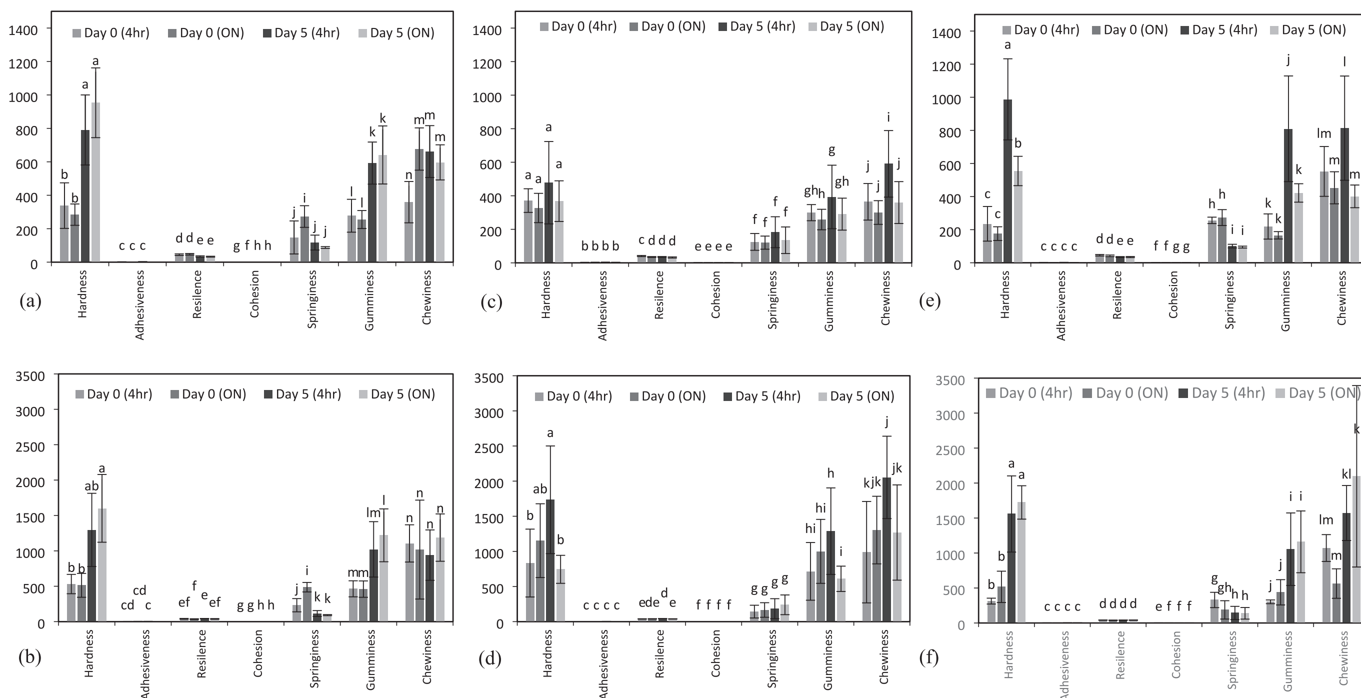

Fig. 3. Sourdough (SD) bread texture profile[a,b]. (a): yeast crumb texture; (b): yeast crust texture; (c): *Lb. rhamnosus* (RBL 102) crumb texture; (d): *Lb. rhamnosus* (RBL 102) crust texture; (e): *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52) + *Lb. rhamnosus* (RBL 102) crumb texture; (f): *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52) + *Lb. rhamnosus* (RBL 102) crust texture.

[a] 4 h: SD bread fermented for 4 h; ON: SD bread fermented for 4 h and refrigerated overnight.

[b] Values for the same quality parameter with different letters differ significantly (α < 0.05).

experienced elevated rate of texture firming as compared to SD1 bread. However, while 4 h and ON bread samples possess similar characteristics on Day 0, ON fermentation continues to display beneficial effects on retarding, and delaying textural and overall quality changes during storage, especially in adhesiveness, gumminess, and chewiness of SD2 bread crumb, based on Fig. 3(e).

Overall, the different variables evaluated (including the length of fermentation time, and the individual or combination strains of SD1 and SD2 LAB cell culture, characterized by their different profiles of enzyme expression), demonstrate jointly the significance of *in situ* LAB incorporation, especially when the LAB culture is previously optimized in producing bread quality improving enzymes, on the resulting texture of SD bread samples during shelf-life. Several studies conducted with a focus on applying both SD technology and *ex situ* enzyme supplementation also agree on that regard. However, these studies have highlighted the potential inactivation of externally added enzymes due to the typically low pH environment within SD (Galle, 2013; Komlenić et al., 2010), which makes *in situ* LAB incorporation with intrinsic optimal enzyme abilities advantageous. Nevertheless, according to Katina et al. (2006), the co-application of SD and a mixture of enzymes (α-amylase, xylanase, and lipase) demonstrates statistically significant efficiency in improving and maintaining crumb softness both on Day 0 and Day 6, compared to bread with either exclusive SD starter incorporation or with only enzyme mixture addition. The low degree of staling reported in the study is believed to be majorly contributed by the amylolytic action, where α-amylase breaks the starch polymers that connect different crystalline regions, making the crumb less prone to starch retrogradation and amylopectin crystallization, influencing moisture availability in the meantime (Taglieri et al., 2021). The interaction with other enzymes within the crumb structure is important as well in mitigating the firming and staling of bread, by participating in water distribution and maintaining hydration level throughout the loaf (Fadda et al., 2014). According to Cevoli, Gianotti, Troncoso, & Fabbri (2015), the addition of exogenous enzymes, including α-amylase, xylanase, and lipase, can exert synergistic actions together with yeasts and LAB, by improving mechanical property and shelf-life of flatbread samples. Furthermore, the presence of both SD and enzymes helps with maintaining adequate water activity, and limiting starch retrogradation and water mobility during the storage period.

The strong standalone influence of SD on bread texture is a source of continuously renewed interest, in increasing quality and shelf-life, and delaying staling. However, the utilization of LAB culture in SD, is strongly strain-specific (Galli et al., 2019). In particular, LAB strains with intrinsic proteolytic and amylolytic activities are considered the most effective in delaying staling (Galle, 2013). For LAB strains with EPS-producing abilities, depending on the type of EPS being produced, they have the additional possibility for anti-staling actions (Wang et al., 2019). Furthermore, stabilizing effect towards moisture preservation and crumb softness, has also been recorded for bread samples prepared with SD culture containing *Lb. plantarum*. Such observation is suggested to be the outcome of complex interaction existing within the loaves, involving the LAB cell culture and their cell lysates, both during the breadmaking process and subsequently during storage life. They act desirably and continuously together on the major and minor fractions within the crumb and crust structures, including gluten, starch, non-starch polysaccharides, and lipids (Taglieri et al., 2021).

Previous studies have recorded significant rheological and pH change in dough after prolonged fermentation. This leads to a dramatic reduction in firmness and elasticity of bread loaves, which is in alignment with findings from the present study, and has important implications on reducing staling and extending bread shelf-life (Abedfar & Sadeghi, 2019). Prolonged fermentation facilitates greater acidification within the dough, while allowing the LAB cells to work optimally, especially those efficient in expressing proteolytic and amylolytic enzymes, to degrade and alter the gluten and starch network in a more extensive manner (Abedfar & Sadeghi, 2019). The increased production of carbon dioxide due to the prolonged fermentation can also enhance leavening of dough and bread that is important in elevating loaf volume and delaying staling (Fadda et al., 2014; Galle, 2013).

3.2.2. LAB cell-lysate-enriched bread texture during shelf-life

Dough samples made with respective lysates originating from concentrated C-MD1 (*Lb. delbrueckii* subsp. *bulgaricus*), C-MD2 (*Lc. lactis* subsp. *diacetylactis* and *Lb. rhamnosus*) LAB cells, in addition to the control dough MD0 made without LAB cell lysates, are subjected to four hours of fermentation, before being baked into bread loaves. MD2 dough samples are exclusively enriched with 1 % flour weight of yeast β-glucan. The isolated effects of LAB cell lysates are thereby evaluated, discerned based on their respective enzyme and EPS expression profile, both post-baking and during shelf-life. The texture behavior of the MD bread samples can be found under Fig. 4.

Based on Fig. 4(a), MD0 bread loaves experience steady rate of crumb texture firming at the evaluated intervals, graphically indicated through continuous increase in value for hardness, gumminess, and chewiness attributes. Meanwhile, crumb resilience, cohesion, and springiness stabilize in later stages of shelf-life, after an initial reduction. Crust texture of MD0 in Fig. 4(b) demonstrates a similar tendency. Most drastic texture transformation occurs during the first 6 days of shelf-life, specifically for hardness, springiness, gumminess, and chewiness.

Different aspects of texture measurements of MD1 bread samples are recorded at higher values, both initially and during shelf-life, as compared to MD0. However, statistical analysis presented in Fig. 4(c) and Fig. 4(d) indeed shows an adequate influence of LAB cell lysates in reducing the effect of storage time on bread texture quality. Bread crumb changes in hardness, adhesiveness, springiness, gumminess, and chewiness levels are statistically indistinguishable either after Day 6 or Day 10 of shelf-life in MD1 samples. Similarly for bread crust, the majority of all analyzed texture components experiences little to almost no significant changes from Day 6 onwards.

As comparing to MD1 and MD0 breads, MD2 bread crumb and crust samples both undergo statistically notable changes throughout the whole period of shelf-life, especially regarding hardness, gumminess, and chewiness. However, MD2 bread crumb adhesiveness, resilience, and springiness are well maintained, as well as adhesiveness and cohesion of MD2 bread crust to a certain extent according to Fig. 4(e) and Fig. 4(f).

Numerous studies have reported on the positive effect of enzyme mixture, that exerts different functions against texture staling of wheat bread, concerning α-amylase, xylanase, lipase, and protease (Dai & Tyl, 2021; Kieliszek et al., 2021; Taglieri et al., 2021). The effect of glucose oxidase and laccase on staling retardation that concerns this study has also been mentioned and researched upon individually, however their synergistic effect as part of a mixture with other bread improving enzymes is yet to be fully explored (Dai & Tyl, 2021; Fadda et al., 2014). The joint effects by xylanase and α-amylase, in improving dough extensibility, against bread firming and maintaining texture qualities, have been highlighted in numerous studies, in samples evaluated freshly baked and after a few days of storage (Dai & Tyl, 2021; O'Shea et al., 2016). The enzyme mix is considered effective when applied at an adequate dosage, in a wide range of bakery products including pan bread, flatbread, and in products made with wheat flour or blends of wheat flour with other flours (Dai & Tyl, 2021; Kim & Yoo, 2020). Similar effects are reported with the supplementation of both gluten-degrading protease and α-amylase, that preserve gumminess and chewiness, while diminishing bread staling during shelf-life (Dai & Tyl, 2021).

While preliminary baking tests following the SD method described by Dong et al. (2024) indicated significant improvement in bread volume and texture qualities with both *in situ* and *ex situ* EPS inclusion, the MD bread samples in the current study, expected to contain EPS through expression of EPS-producing strain *Lc. lactis* subsp. *diacetylactis* (RBL 37), along with *ex situ* yeast β-glucan supplementation, did not exhibit

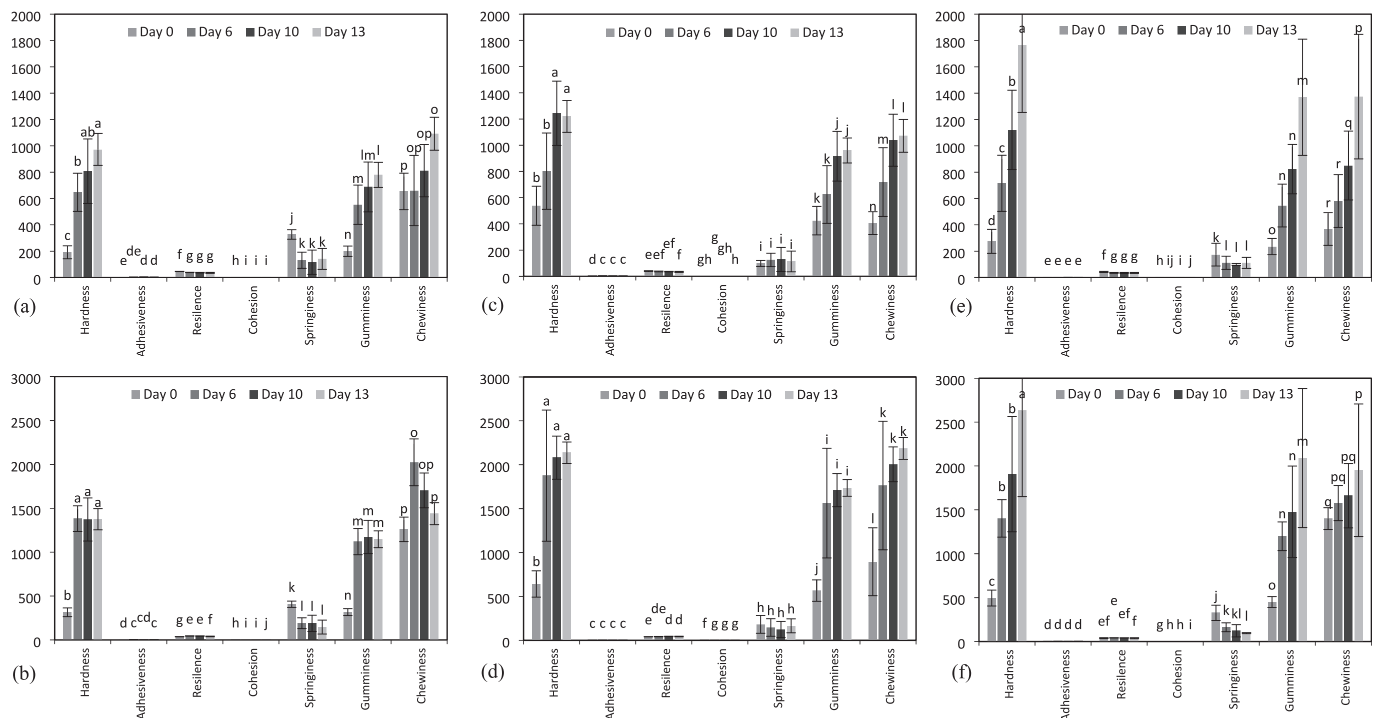

Fig. 4. LAB cell-lysate-enriched (MD) bread texture profile[a]. (a): yeast crumb texture; (b): yeast crust texture; (c): *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52) crumb texture; (d): *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52) crust texture; (e): *Lc. lactis* subsp. *diacetylactis* (RBL 37) + *Lb. rhamnosus* (RBL 102) crumb texture; (f): *Lc. lactis* subsp. *diacetylactis* (RBL 37) + *Lb. rhamnosus* (RBL 102) crust texture.

[a] Values for the same quality parameter with different letters differ significantly ($\alpha < 0.05$).

notable improvements in freshly baked samples or in retarding rate of texture firming during shelf-life. EPS inclusion in bread products is well-established to have anti-staling and shelf-life prolongation benefits, depending strongly on the structure and type of EPS utilized (Lynch et al., 2018). This effectiveness can be attributed to their innate water-binding ability, that restricts migration of free water molecules from crumb to crust, and limits the extent of starch crystallization (İspirli et al., 2020; Taglieri et al., 2021). Enhanced initial crumb softness has also been reported with EPS application (Lynch et al., 2018). Similarly, quality preservation attributes have also been reported specifically for β-glucan application (İspirli et al., 2020; Xu et al., 2021). In alignment with the current study, previous studies have suggested that *in situ* ways of EPS supplementation are more effective than *ex situ* incorporation (İspirli et al., 2020), and most texture protective effects of EPS during shelf-life have been reported in SD breads with *in situ* incorporation method (Lynch et al., 2018; Taglieri et al., 2021). It has also been suggested that β-glucan can be extensively depolymerized with direct EPS addition into dough, as compared to doughs made using the SD method. The depolymerization leads to inadequate preservation of the β-glucan structure and loss of efficiency of its physio-chemical properties, including water binding capabilities among other texture modifying actions, that are associated closely with its anti-staling effects (Moriartey et al., 2011).

After extensive surveying of the literature, the mechanism and potential synergism combining both enzymes and EPS, on delaying bread texture firming, remain largely unclear. Interactions between enzymes and hydrocolloids have also been investigated due to the hydrocolloid characteristics of EPS. Based on research conducted by Gujral et al. (2004) on rice flour chapaties, while there is a reduction in starch retrogradation rate observed in the co-application of α-amylase and hydrocolloids (for example: guar gum, xanthan, locust bean gum), the benefit is minor as compared to exclusive use of α-amylase.

3.3. Evolution of bread flavor during shelf-life

It is a common perception that bread flavor is noticeably altered after a few days of storage as compared to freshly baked bread. In particular, there is a reduction in number and variety of key aromatic compounds presentin both bread crumb and crust, including 2,3-butanedione, and 2-acetyl-1-pyrroline, accompanied generally by the appearance and increase of "off-flavor" compounds during shelf-life (De Vuyst et al., 2021; Jensen et al., 2011; Prost et al., 2020). These changes, together with crumb firming and textural deterioration, are fundamental contributors to the loss of bread freshness and overall consumer acceptance (Pétel et al., 2017; Pico et al., 2015; Prost et al., 2020). The flavor profile analyzed using gas chromatography–mass spectrometry (GC–MS) facilitates evaluation of the intensity and type of key bread flavors, as well as a deeper understanding of the intrinsic chemical alteration that occurs in freshly baked bread and during the shelf-life period (*Supplementary Fig. 1)*. The result present below demonstrates successfully the efficiency of LAB-based bio-ingredients in bread flavor preservation during shelf-life, which confirms the validity of tis study's hypothesis.

Flavor profile is established for SD crumb and crust of sample sets SD0 (culture of yeast), SD1 (culture of yeast and *Lb. rhamnosus*), and SD2 (culture of yeast, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. rhamnosus*). The key variables impacting bread flavor that are investigated include: difference in LAB culture utilized in SD1 and SD2 as compared to yeast culture SD0, the influence of either 4 h or ON fermentation, and the duration of shelf-life. 25 flavor compounds in total have been identified, including 2 alkanes, 4 acids, 6 alcohols, 2 ketones, 7 aldehydes, 1 pyrrole derivative, and 3 esters (*Supplementary Fig. 2)*. Ethanol is excluded from all calculations due to its oversaturated concentration. Among the identified volatiles, 2-acetyl-1-pyrroline is considered as a primary crust odorant (Cho & Peterson, 2010). Meanwhile, 2,3-butanedione is a key product generated during bread baking through Maillard reaction and caramelization. Both processes make significant contribution to crust aroma and color (Prost et al., 2020).

According to *Supplementary Fig. 3(a)* and *Supplementary Fig. 3(b)*, there is a limited number of flavor compounds identified in the SD0 bread crumb and crust, chiefly includes 2-methyl-1-propanol, propanoic acid, ethyl acetate, 2,3-butanedione, acetic acid, octane, and hexanal. Their levels are all extremely low in both 4 h and ON SD0 bread crumb and crust, on both Day 0 and Day 5. The initial concentration on Day 0 of 2,3-butanedione is recorded at 0.014 ppm and 0.019 ppm, in the crust of 4 h and ON SD0 bread respectively. On Day 5, 2,3-butanedione is reduced to 0.003 ppm in 4 h SD0 bread crust, and 0.005 ppm in ON SD0 bread crust.

In comparison, the crumb and crust of SD1 bread samples, which correspond to Fig. 5(a) and Fig. 5(b), evoke a more comprehensive and balanced array of SD flavor compounds, both qualitatively and quantitatively. All 25 flavor compounds chemically identified within the scope of the SD experiment in this study are present in SD1 bread samples. Meanwhile, the volatiles are present much more prominently than in SD0 samples, with the major compounds in SD1 bread, excluding acetic acid, reaching as high as 0.6 ppm for both crumb and crust. The notable improvement, especially evident in ON bread samples, runs parallel with the texture assessment of SD1 in relation to SD0 samples as previously discussed. There is a consensus shared among various publications that accumulation of metabolites, including volatile and aroma compounds, is generally favored when dough is subjected to longer fermentation (Arora et al., 2021; Gunduz et al., 2022). The fermentation stage is a crucial step during which important bread aromas are generated. Therefore, a significant reduction in fermentation time can have a strong influence in the resulting bread aroma profile (Liu et al., 2020; Prost et al., 2020). By juxtaposing Fig. 5(a) and Fig. 5(b), the SD1 bread samples follow the general tendency reported in literature, where bread crust possesses more flavor compounds than crumb (Chiavaro et al., 2008; Prost et al., 2020), due to the extensive non-enzymatic Maillard reaction taking place during baking (Chiavaro et al., 2008). The common desirable sensory notes for bread (fruity, fermented, roasty, and acidic), can be supplied by 2,3-butanedione, 3-methylbutanal, methional, and 2-acetyl-1-pyrroline, among many others (De Vuyst et al., 2021; Pico et al., 2015; Prost et al., 2020). The majority of the detected compounds are found more prominently in ON bread than in 4 h bread, for both SD bread crumb and crust. SD2 bread samples as detailed in Fig. 5 (c) and Fig. 5(d), display a similar flavor profile as SD1 breads, in terms of both amplitude and variety of compounds characterized. However, an additional different LAB strain in SD2 bread does not appear to have a remarkable effect in diversifying and intensifying the crumb and crust flavor, nor in maintaining the existing flavor during shelf-life, as compared to SD1 containing solely *Lb. rhamnosus* LAB cells (Pétel et al., 2017; Prost et al., 2020; Yan et al., 2019). Meanwhile, LAB incorporation still demonstrates a staggering advantage over control bread SD0, enhanced still by ON fermentation. ON fermentation in SD2 breads accentuates the pleasant sweet, malty, roasty, and earthy notes contributed by 3-methylbutanal and methional in bread crumb, and furfural in bread crust (Pétel et al., 2017; Prost et al., 2020). ON fermentation also appears to alleviate the impact of lipid oxidation in SD2 bread during shelf-life, demonstrated as a significant reduction in concentration of 2-methyl-1-propanol, 2-methyl-1-butanol, and hexanal on Day 5, that usually increase during shelf-life, and can participate in the generation of undesirable stale and off-flavor notes. However, there is notable alteration in flavor composition on Day 5 as compared to Day 0 for SD2 samples, particularly for 2,3-butanedione.

The change in bread texture during storage as previously discussed occurs in parallel with the alterations of aroma profile. Crumb structural components, such as gluten and starch, are capable of entrapping volatiles, thereby delaying the depletion of important bread key odorants to a certain extent (Chiavaro et al., 2008; Cho & Peterson, 2010). In which case, the structure modifying, texture preservation, and anti-staling effects offered by the different variables of interest in this study (SD incorporation, length of fermentation time, *in situ* or *ex situ* application of LAB cell lysates, including EPS and quality-improving enzymes), can

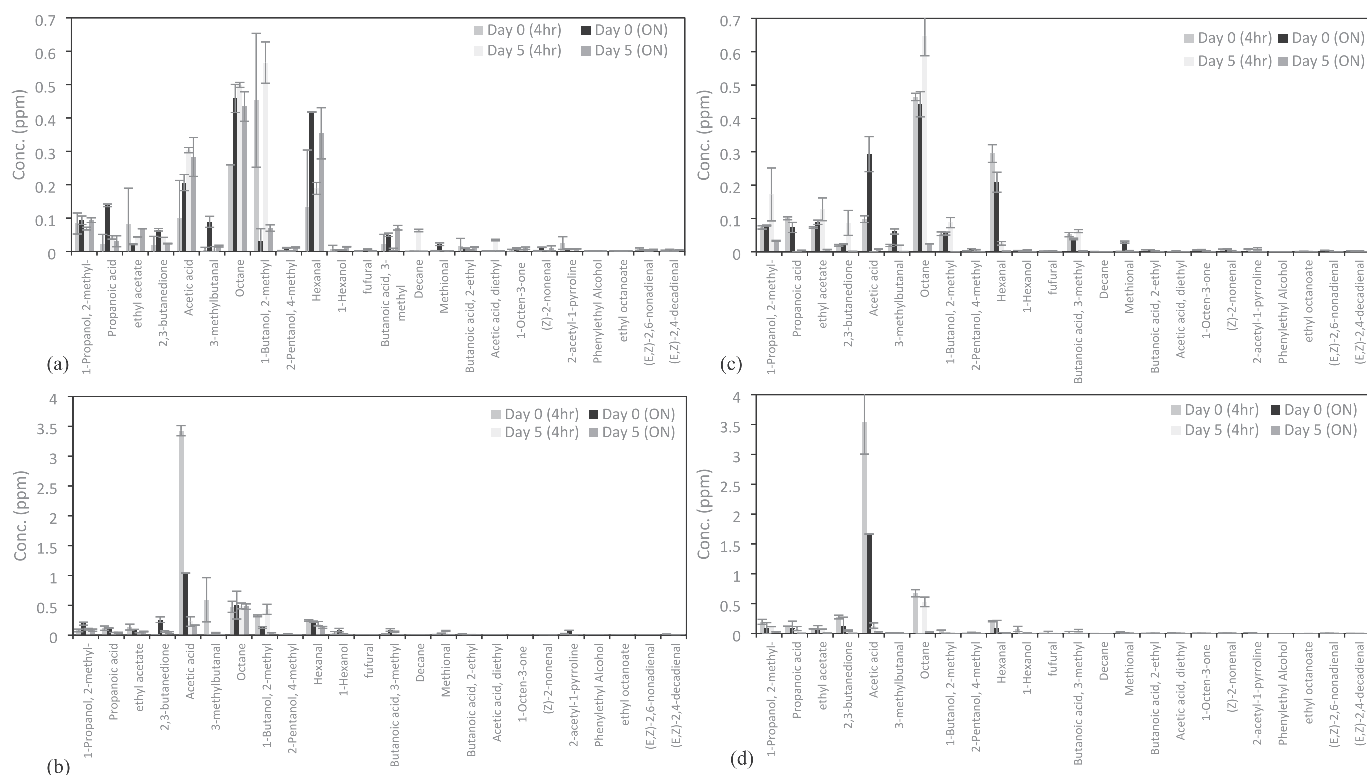

Fig. 5. Sourdough (SD) bread flavor profile[a]. (a): *Lb. rhamnosus* (RBL 102) crumb flavor; (b): *Lb. rhamnosus* (RBL 102) crust flavor; (c): *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52) + *Lb. rhamnosus* (RBL 102) crumb flavor; (d): *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52) + *Lb. rhamnosus* (RBL 102) crust flavor.

[a] 4 h: SD bread fermented for 4 h; ON: SD bread fermented for 4 h and refrigerated overnight.

consequently supply comprehensive protection on bread products during shelf-life, including maintenance of flavor profile. In particular, SD fermentation can significantly reduce the concentration of malodorous aroma compounds primarily generated through lipid oxidation during shelf-life, with some LAB capable of converting lipid oxidation compounds into alcohols, depending on the specific microbial and metabolic profile of these LAB strains (Pétel et al., 2017). Furthermore, SD fermentation permits intensification of the initial release of desirable bread volatiles that is especially apparent with prolonged fermentation, that can diminish the likelihood of complete depletion of typical aroma associated with fresh bread during storage period, thereby making the off-flavor aromas less apparent and substantial (Pétel et al., 2017; Pico et al., 2015; Prost et al., 2020).

21 flavor compounds in total were recognized for MD bread crumb and crust sample sets (MD0 (yeast), MD1 (*Lb. delbrueckii* subsp. *bulgaricus*), and MD2 (*Lc. lactis* subsp. *diacetylactis* and *Lb. rhamnosus*). MD1 and MD2 are supplemented with corresponding LAB cell lysates and compared with MD0. The range of flavors identified comprised of 2 alkanes, 3 acids, 3 alcohols, 2 ketones, 7 aldehydes, 1 pyrrole derivative, and 3 esters *(Supplementary Fig. 4)*. Similar to SD flavor profiling, ethanol is also excluded from discussion.

A series of flavor compounds are identified both on the day of baking and during shelf-life for MD0 bread according to Fig. 6(a) and Fig. 6(b). The high level of acetic acid and propanoic acid present especially at the beginning of shelf-life, can be explained by the addition of various preservative agents to prevent mould growth. Ample concentration of 2-methyl-1-propanol, hexanol, and the malodorous hexanal, in both crumb and crust, can provide insight into the progression of lipid oxidation during bread storage (Jensen et al., 2011). The concentration of hexanal in particular, is correlated negatively with the acceptance of bread aroma (Jensen et al., 2011; Pico et al., 2015). Several other odorants are reported, but no discernible pattern of their behavior in relation to the length of storage time can be established.

The incorporation of LAB cell lysates in MD1 bread, as presented in Fig. 7(a) and Fig. 7(b), does not appear to have a notable influence towards enhancing the bread flavor profile as anticipated. Similar to MD0, desirable bread odorants including 2,3-butanedione, 3-methylbutanal, furfural, and 2-acetyl-1-pyrroline, are detected at minor and negligible levels both on Day 0 and thereafter.

MD2 bread samples in Fig. 7(c) and Fig. 7(d), are assessed in order to investigate the effect of *ex situ* EPS incorporation towards flavor generation post-production and during shelf-life, by applying both yeast cell wall beta-glucan and LAB cell lysates prepared from the EPS-positive strain *Lc. lactis* subsp. *diacetylactis* (RBL 37). Exclusive EPS supplementation in MD2 breads, along with adding double the amount of LAB cell lysates compared to MD1 bread, does not seem.

to significantly enhance the complexity and potency of crumb and crust flavors, nor stability of existing flavors under storage conditions. Bread key volatiles previously detailed remain low in both MD2 crumb and crust.

While the *ex situ* mode of LAB cell lysate incorporation in the present study does not appear to have a pronounced influence on enhancing bread flavor profile as expected, the introduction of microbial enzymes, in addition to endogenous enzymes naturally present in flour, can supposedly intensify and diversify the generation of bread flavor compounds, by providing a pool of precursors ready for fermentative and

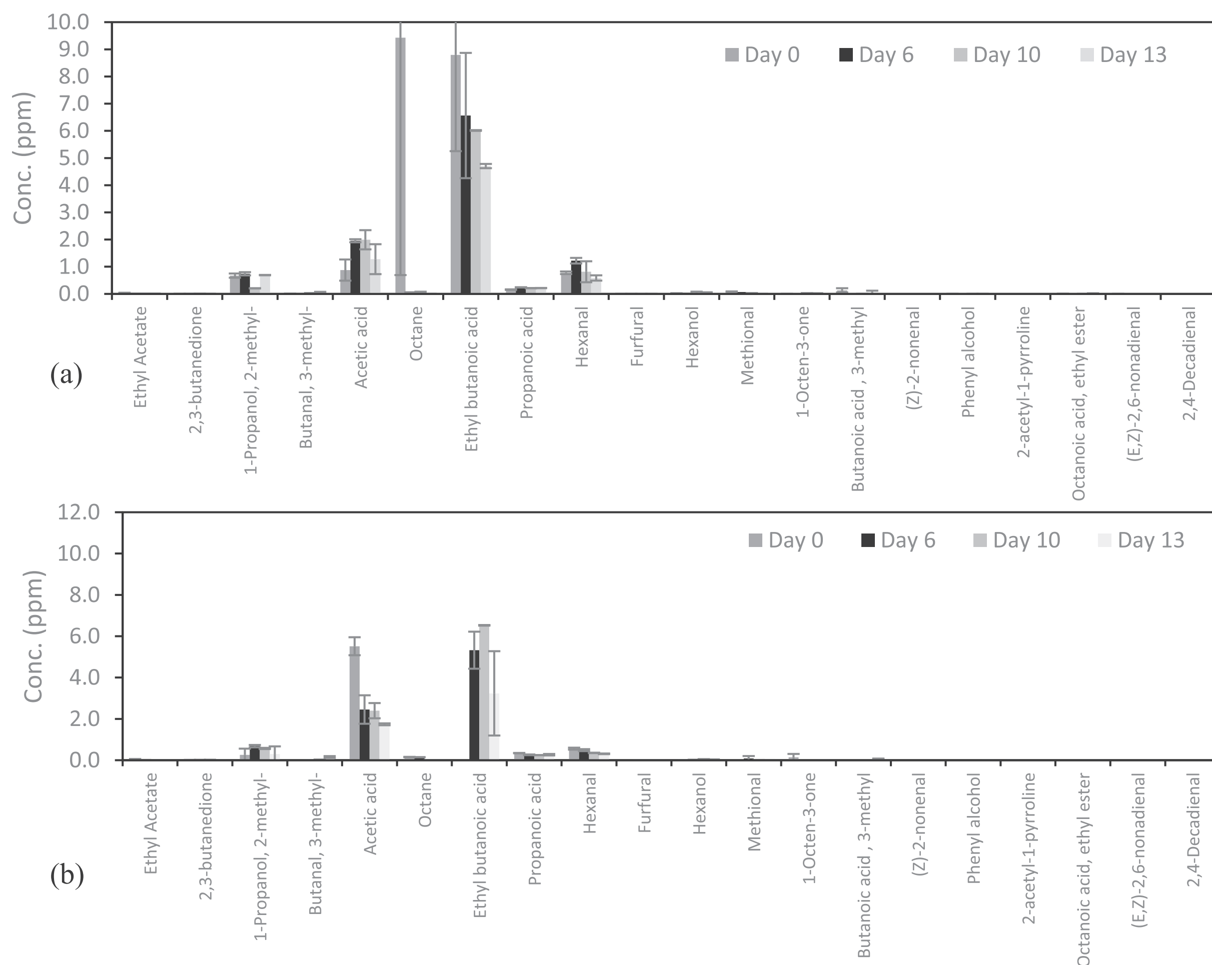

Fig. 6. LAB cell-lysate-enriched (MD) bread flavor profile. (a): yeast crumb flavor; (b): yeast crust flavor.

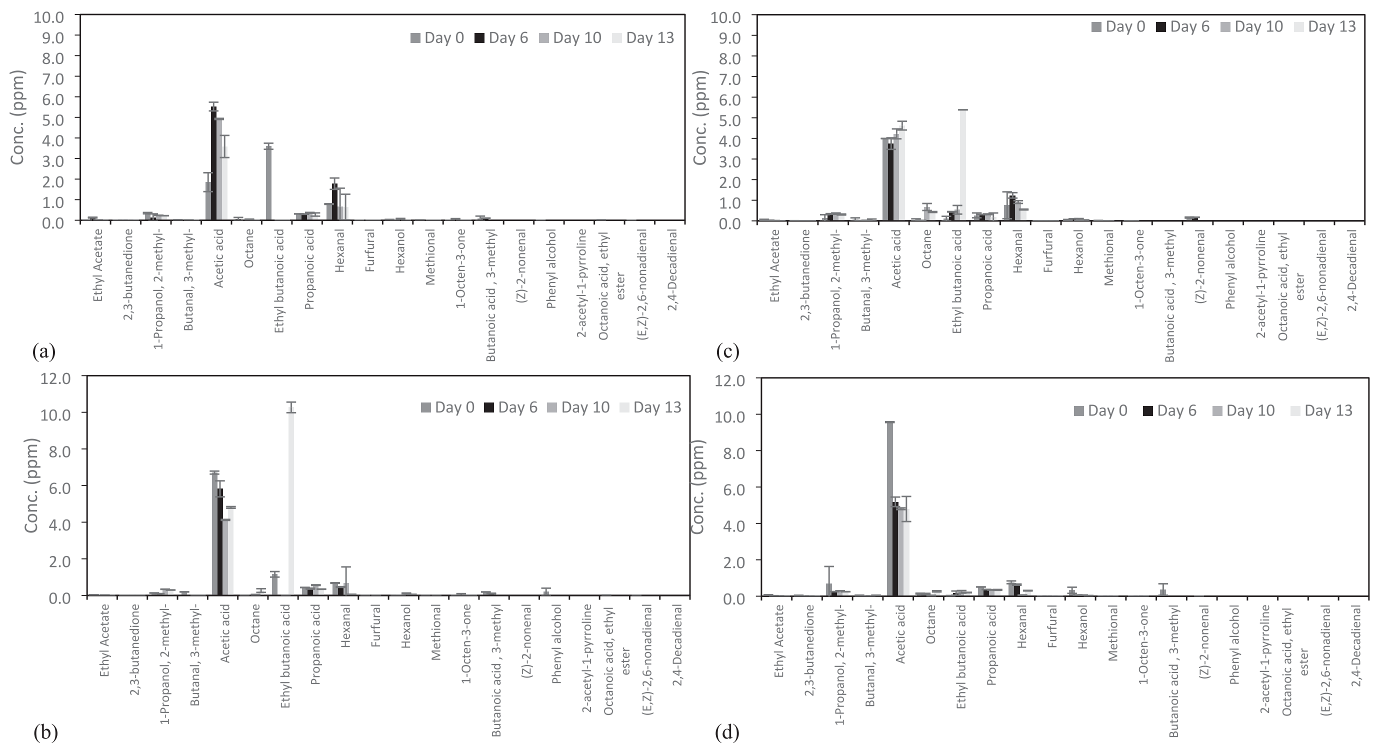

Fig. 7. LAB cell-lysate-enriched (MD) bread flavor profile. (a): *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52) crumb flavor; (b): *Lb. delbrueckii* subsp. *bulgaricus* (RBL 52) crust flavor; (c): *Lc. lactis* subsp. *diacetylactis* (RBL 37) + *Lb. rhamnosus* (RBL 102) crumb flavor; (d): *Lc. lactis* subsp. *diacetylactis* (RBL 37) + *Lb. rhamnosus* (RBL 102) crust flavor.

thermal reactions (Pico et al., 2015; Prost et al., 2020). In particular, hydrolytic actions upon major structural component of both starch and gluten, carried out respectively by glycosyl hydrolases (including α-amylase and xylanase) and proteases along with peptidases, improve availabilities of low-molecular-weight sugars and free amino acids as well as peptides, that act as fermentation substrates, participate in Maillard reaction, and consequently have an influence in the final bread aroma compounds derived from them (Pico et al., 2015; Prost et al., 2020). Presence of oxidases, such as glucose oxidase and laccase, are beneficial in improving bread textural quality as discussed before. However, they may lead to undesirable off-flavor generations due to the oxidative reactions they catalyze (Chiavaro et al., 2008; Pico et al., 2015). Furthermore, the presence of EPS can promote additional metabolic and flavor enhancing activity, by increasing production of lactate, acetate, and ethanol, as well as supplying a reservoir for subsequent aroma generation processes to take place (Di Monaco et al., 2015). Enhanced acidity has also been reported for dough enriched with *endo*-xylanase, α-amylase, and EPS, providing additional flavor enhancing effects (Di Monaco et al., 2015).

4. Conclusion

LAB based bio-ingredients offer comprehensive and promising advantages on dough and bread quality attributes in freshly baked bread, and provide bio-protection during shelf-life. Between *in situ* and *ex situ* mode of LAB incorporation, the SD breads with *in situ* supplementation, especially of *Lb. rhamnosus*, produce desirable initial bread texture and flavor quality. These quality attributes that are important for the perception of bread freshness during shelf-life, were maintained at an adequate level during shelf-life, as compared to the control sample without LAB incorporation. The prolongation of the fermentation period also seems to amplify that benefit. Future optimization of the *in situ* approach can be directed towards addressing the time limitation concern typically associated with bread manufacture in an industrial setting, by improving the efficiency and adaptability of these functional LAB cells. For *ex situ* incorporation, the quality-enhancing influence on dough and bread, by inclusion of LAB cell lysates and external sources of EPS, can inspire future studies on studying the interactions among dough structural components and quality improvers, and how to better integrate these quality-enhancing ingredients to achieve the most desirable effect.

CRediT authorship contribution statement

Yining Dong: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Elham Chidar:** Methodology, Formal analysis. **Salwa Karboune:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101857.

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