New bread formulation with improved rheological properties and longer shelf-life by the combined use of transglutaminase and sourdough

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ABSTRACT

The combined use of the protein reticulating enzyme transglutaminase (TGase) and a selected microbial consortium of *Lactobacillus sanfranciscensis* and *Candida milleri* for improving the rheological properties, aroma, and shelf life of a bakery product was evaluated. A microbial TGase, showing the highest activity over a wide temperature range on different protein substrates, was selected among different types. Results showed that this TGase was able to produce isodipeptide bonds, especially in the gluten fraction, leading to the formation of protein aggregates, which improved the structure of a sourdough bakery product. The microbial TGase in combination with sourdough exhibited a positive synergistic effect allowing the production of flavor enriched bread, with rheological properties similar to those of standard bread.

1. Introduction

Bread is a fundamental food in the Western world and it is generally viewed as a perishable commodity, due to its fast decrease of freshness features and its rapid staling (Minervini, De Angelis, Di Cagno, & Gobbetti, 2014). Lactic acid bacteria (LAB) and yeasts in the form of sourdough have been reported to have positive effects on wheat bread quality and staling (Clarke, Schober, & Arendt, 2002; Corsetti et al., 2000; Crowley, Schober, Clarke, & Arendt, 2002) as they are responsible for the capacity of dough to leaven, while acidifying it (De Vuyst & Neysen, 2005). Traditional sourdough obtained with selected microorganisms is able to in crease bread shelf life by delaying staling (Chavan & Chavan, 2011) and improve bread properties through enhancing its nutritional value, taste, and aroma profile (Arendt, Ryan, & Dal Bello, 2007; Hansen & Schieberle, 2005; Poutanen, Flander, & Katina, 2009). However, the use of LAB may affect the rheology of leavened bakery products through a strain dependent proteolytic activity (Gobbetti, Smacchi, & Corsetti, 1996). These rheological properties, besides gas retention, depend on gluten proteins, composed of extensible, viscous gliadins and rigid, elastic glutenins. Chemical agents or

Abbreviations used: acTGase, Activa WM transglutaminase; AEC, 3-amino-9-ethylcarbazole; ANOVA, analysis of variance; AtPng1p, *Arabidopsis thaliana* pep-tide *N*-glycanase; CFU, colony forming unit; C, control; Cd, control dough; Cb, conventional bread; DY, dough yield; DMC, *N*′, *N*′-dimethyl casein; FN, fibronectin; SPME-GC-MS, gas chromatography-mass spectrometry coupled with solid phase micro-extraction; HRP, horseradish peroxidase; LAB, lactic acid bacteria; PC, principal component; PCA, principal component analysis; Sd, selected sourdough; Sb, sourdough bread; TGase, transglutaminase; TPA, texture profile analysis; MCFA, medium-chain fatty acid; RT, room temperature; SDS-PAGE, sodium dodecyl sulphate poly acrylamide gel electrophoresis; VCs, volatile compounds.

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cross linking enzymes, such as glucose oxidase, peroxidase, or transglutaminase (TGase), have been reported to improve dough handling properties and to increase fermentation stability, and loaf volume (Caballero, Gómez, & Rosell, 2007; Steffolani, Ribotta, Perez, & Leon, 2010). In particular, TGase (EC 2.3.2.13) is an important enzyme for the food industry (Basman, Koksel, & Ng, 2002) as it catalyses the formation of protein cross links resulting in extensive nets (Nonaka et al., 1989). The formation of protein polymers, as a result of TGase activity, can modify the rheological properties of gluten (Koksel, Sivri, Ng, & Steffe, 2001) and allow the transformation of a very weak gluten into a very strong one (Larre et al., 2000). In previous studies, the positive effects of TGase application on wheat based baked products have been described (Renzetti, Behr, Vogel, & Arendt, 2008), (Gerrard et al., 2000). The effects of TGase on empirical rheological properties of dough (Basman et al., 2002; Marco, Perez, Ribotta, & Rosselli, 2007) and on the formulation of sourdough (Arendt et al., 2007; Clarke et al., 2002) in order to obtain good quality bread have been described. To date, however, the combined use of the two biological agents, i.e., TGase and sourdough, has never been reported.

In the present work, we tested the possibility of improving bread quality through the combined use of sourdough and a protein reticulating enzyme. TGase was selected in order to improve rheology (Gerrard et al., 1998), while sourdough based on *Lactobacillus sanfranciscensis* and *Candida milleri* was chosen for its ability to improve the aroma profiles and extend the shelf life of the final product (Scarnato et al., 2016; Vernocchi et al., 2008). *L. sanfranciscensis* is a key organism for sourdough acidification and produces aroma precursors (Gobbetti et al., 1996; Ganzle, Vermeulen, & Vogel, 2007), while *C. milleri* is able to grow in as sociation with hetero fermentative LAB, enhancing the accumula tion of specific aroma compounds, including alcohols, lactones, and medium chain fatty acids (MCFAs) (Gobbetti, 1998; Ganzle, Ehmann, & Hammes, 1998). Results show that the combination of a protein reticulating enzyme and sourdough on wheat bread produced a positive synergistic effect.

2. Materials and methods

2.1. Materials

Straight grade wheat flour was provided by Barilla S.p.A.(Parma, Italy). The TGases tested in this study came from different sources: (i) Activa® WM (acTGase), from *Streptoverticillum mobar aense*, was purchased from Ajinomoto (specific activity: >0.1 U/mg powder preparation whose composition is 1% enzyme and 99% maltodextrin), Mesnil Saint Nicaise, France, (ii) a recombinant microbial TGase (zTGase) was purchased from Zedira (specific ac tivity: >25 U/mg, Darmstadt, Germany), (iii) a mammalian TGase, from guinea pig liver, was purchased from Sigma Aldrich (specific activity: >2.4 U/mg, Milan, Italy), and (iv) a recombinant TGase of plant origin, the *Arabidopsis thaliana* peptide *N* glycanase (AtPng1p), was purified as previously described (Della Mea, Caparros Ruiz, & Rigau, 2004) (specific activity: > 0.5 U/mg).

All reagents and solvents (unless otherwise indicated) were of the highest purity and were obtained from Sigma Aldrich (Milan, Italy).

2.2. Sourdough preparation

Sourdough was prepared by a two step fermentation process using *L. sanfranciscensis* strain LSCE1 and *C. milleri* strain PFL44, both belonging to the Department of Agricultural and Food Science, University of Bologna (Italy). The dough was prepared by mixing wheat flour and water to reach a dough yield (DY) of 220. Exposure to osmotic stress was performed by adding sucrose (40% of the final dough volume) to the water. Fermentation was performed in a fermentor (BioFlo/CelliGen® 115, New Brunswick, Eppendorf) as previously described (Scarnato et al., 2016). The sourdough ob tained was used for the preparation of bread in association or not with TGase.

2.3. Enzymatic treatment with TGase

In order to induce protein cross links, different amounts of TGase (0.5, 1, 2, and 5 U/g flour) were added to the wheat dough (Cd) obtained using *Saccharomyces cerevisiae* as a conventional leavening agent and the sourdough (Sd) made with the selected microbial consortium described above. The enzyme was mixed to the flour for 15, 60 or 90 min at different temperatures, from 4 to 37 °C.

Cross linking was evaluated by protein extraction and separa tion using 15% sodium dodecyl sulphate polyacrylamide gel elec trophoresis (SDS PAGE). Total proteins were extracted under reducing conditions using the buffer described by (Marco et al., 2007). Albumins/globulins (F1), prolamins (F2), and glutelins (F3) were extracted following a sequential extraction method using different solvents (Marco et al., 2007). Globulins 7S and 11S were prepared and purified as previously described (Thanh, Okubo, & Shibasaki, 1975). The protein content of the extracts was deter mined by the bicinchoninic acid method (Smith et al., 1985).

2.4. Measurement of TGase activity

TGase specific activity was measured by the conjugation of biotinylated cadaverine to protein substrates as previously described (Lilley, Skill, Griffin, & Bonner, 1998) with slight modifi cations. Protein substrates, such as standard proteins and wheat protein extracts, were covalently attached to the surface of micro plate wells. The level of biotinylated cadaverine incorporation was determined according to an established protocol (Lilley et al., 1998) with 10 mM cystamine replacing EDTA in negative controls. The specific activity was expressed as a 0.1 change in A_{450} per h per mg of TGase used (U/mg prot.).

2.5. Dot blot of enzyme reaction products

Fractions F1, F2, and F3 were treated with TGase and the reac tion products were blotted onto nitrocellulose. The membrane was incubated with Ab3, a monoclonal antibody raised against soluble TGase (Neomarker, Fremont, CA, U.S.A.) and with 81D4, a mono clonal anti Nε (γ glutamyl) lysine antibody (Covalab, Lyon, France), which is a product of the TGase cross linking reaction. Dots were revealed using horseradish peroxidase (HRP) conjugated rabbit anti mouse IgG and 3 amino 9 ethylcarbazole (AEC).

2.6. Bread preparation

Bread was prepared with a bread maker (Deluxe Princess, 152000) using an industrial recipe (wheat flour, water, sugar, salt, baker's yeast, and extra virgin olive oil) to obtain a final volume of about 500 g with DY 150. When Sd was used, it was added at a concentration of about 30% of the final weight of dough; the amount of flour and water was reduced accordingly in order to maintain the same DY. TGase was added at different concentrations (0.5, 1, and 2 U/g flour). Doughs were kneaded for 14 min and fer mented for 20 min. Then, a second kneading of 8 min was per formed, followed by 1 h of fermentation. Dough samples Cd, and Sd with and without TGase were then baked at 180 °C for 30 min in order to obtain conventional bread (Cb) and sourdough bread (Sb).

2.7. Bread empirical rheological properties

Bread mechanical characteristics were evaluated with a Texture Analyzer mod. TA.HDi 500 (Stable Micro System, Godalming, Sur rey, UK) equipped with a P/20 mm aluminium cylinder probe and a 25 kg load cell. For each sample, three slices of 15 mm were cut from the central portion of two different bread loaves. Texture profile analysis (TPA) and stress relaxation tests were performed. The TPA test in a double compression cycle was performed as described earlier (Gàmbaro et al., 2002). Four textural parameters, expressed as hardness, resilience, cohesiveness, and chewiness of the crumb were used as indicators of structural characteristics. The stress relaxation test was performed as described by (Stollman & Lundgren, 1987); texture parameters, such as hardness and springiness, were determined.

2.8. Bread image analysis

The inner portion of bread slices was used for crumb grain features measurements. A digital camera mod. D7000 (Nikon, Shinjuku, Japan) was used to acquire digitalized images of samples placed inside a black box under controlled lighting conditions. The slice images were spatially calibrated using Image Pro Plus v. 6.2 (Media Cybernetics, USA). Grey levels of scanned slices were eval uated by the model in terms of percentage holes and crumb area over the total. The percentage crumb porosity on the total alveo lation of the slice portion was determined. Moreover, crumb morphological features were evaluated including the cell area distribution. Holes were identified, counted and classified into four predefined area classes on the basis of their size (cm2): 0.00025 < class 1 < 0.025; 0.025 < class 2 < 0.25; 0.25 < class 3 < 0.5; 0.5 < class 4 < 1.

Finally, the aspect ratio was calculated by dividing the width by the height of the bread slices (Collar & Angioloni, 2014).

2.9. Bread shelf life evaluation

After a 2 h cooling period, bread samples (Cb and Sb) were introduced in polyethylene plastic bags in ordinary atmosphere, sealed and stored at room temperature (RT) (24 ± 2 °C) for 15 days without preservatives. Samples were monitored daily to check for the presence of spoilage microflora (molds and Bacillus spp.); mi crobial growth was tested by plate counting after 7 and 15 days. Analyses were performed on Malt Extract Agar (Oxoid, Basingstoke, UK) for molds and on Plate Count Agar (Oxoid, Basingstoke, UK) for Bacillus spp.

2.10. SPME Gas Chromatography Mass Spectrometry analysis

Volatile compounds (VCs) were monitored by using Gas Chromatography Mass Spectrometry coupled with solid phase micro extraction (SPME GC MS). Doughs (Cd and Sd) and baked samples (Cb and Sb) were placed in sterilized vials and 10 ml of 4 methyl 2 pentanol (final concentration 33 mg/kg) were added as the internal standard. Samples were pre equilibrated for 10 min at 47 °C and then a fused silica fibre, covered with divinylbenzene/ carboxen/polydimethylsiloxane (DVB/CAR/PDMS StableFlex, Supelco, Steiheim, Germany), was introduced in the head space for 40 min. Molecules were detected using the method described by (Montanari et al., 2014). Identification was based on the compari son of mass spectra with those of NIST and Wiley databases. VCs were expressed as a percentage of the total peak area. Identification of MCFAs was also confirmed by comparing their retention times with those of pure compound mixtures (e.g., BAME Mix, Sigma Aldrich, Italy). Only identified compounds reaching concentrations above 0.2% of the total peak area were used for subsequent Prin cipal Component Analysis (PCA) according to (Patrignani et al., 2016). However, a preliminary one way analysis of variance (ANOVA; significance $P \leq 0.05$) was performed to confirm that the excluded peaks were not significant for sample characterization.

2.11. Statistical analysis

The data are the means of three replicates and are expressed as mean ± standard deviation. The data were examined using one and two way ANOVA with a significant difference of at least 95% according to the Bonferroni post test (GraphPad Prism software), ** $p \leq 0.01$.

Textural data were compared by the Kruskal Wallis test in case of significance with the Levene test ($p < 0.05$) (Statistica 8.0, Stat Soft Inc., Tulsa, OK, U.S.A).

3. Results

3.1. Comparison of TGase activities on different protein substrates

Initially, the four different TGases (acTGase; zTGase; gplTGase and AtPng1p) were tested on well known protein substrates of TGase, such as dimethylcasein (DMC), fibronectin (FN), and some protein fractions present in flours, i.e., 7S and 11S globulins (from soybean), prolamins, gluten, and glutenins from wheat. All the TGases showed activity on the tested substrates. The two TGases of microbial origin (acTGase and zTGase) showed the highest specific activity that reached 4000 U/mg prot (Fig. 1A). gplTGase also showed high activity on these substrates with activities >1000 U/ mg prot. Moreover, the two microbial TGases showed the highest activity on gluten and its components, gliadins and glutenins. When 11S globulins, glutenins, and gliadins were incubated with the various enzymes at different temperatures for 90 min in order to evaluate their cross linking effect, varying degrees of substrate aggregation were visible by SDS PAGE in regions corresponding to high molecular weight (HMW), compared to the same protein not treated with the enzyme (Fig. 1B). At 30 °C, AtPng1p, gplTGase, and acTGase were efficient in the cross linkage of 11S globulin. Both microbial TGases were very effective in reticulating all the tested substrates in a wide range of temperatures (4–37 °C). As acTGase showed the highest activity on the different protein substrates, with major aggregating effects at 30 °C, it was chosen for subse quent experiments.

3.2. acTGase mediated cross linking in dough

As a first attempt to investigate the cross linking activity of acTGase on wheat dough, different enzyme dosages (0.5, 1, 2, and 5 U/g flour) were added. The formation of HMW proteins was dependent on enzyme dosage, mixture time, and water amount. In order to acquire information about the proteins involved in the cross links, different protein fractions (F1, F2, and F3) were extracted from dough. Protein separation by SDS PAGE showed that acTGase, increased the formation of HMW products mostly in F2 and F3 (Fig. 2A, brackets). This was confirmed by the enzyme ac tivity assay, showing that F2 and F3 were the best acTGase sub strates as measured by incorporation of cadaverine (Fig. 2B). Moreover, the immune recognition of the signal corresponding to acTGase increased in supernatants while the appearance of cross links increased in the pellets, as shown in dot blot experiments. F2 showed cross linked products at the lowest acTGase concen tration (0.5 U/g flour) (Fig. 2C).

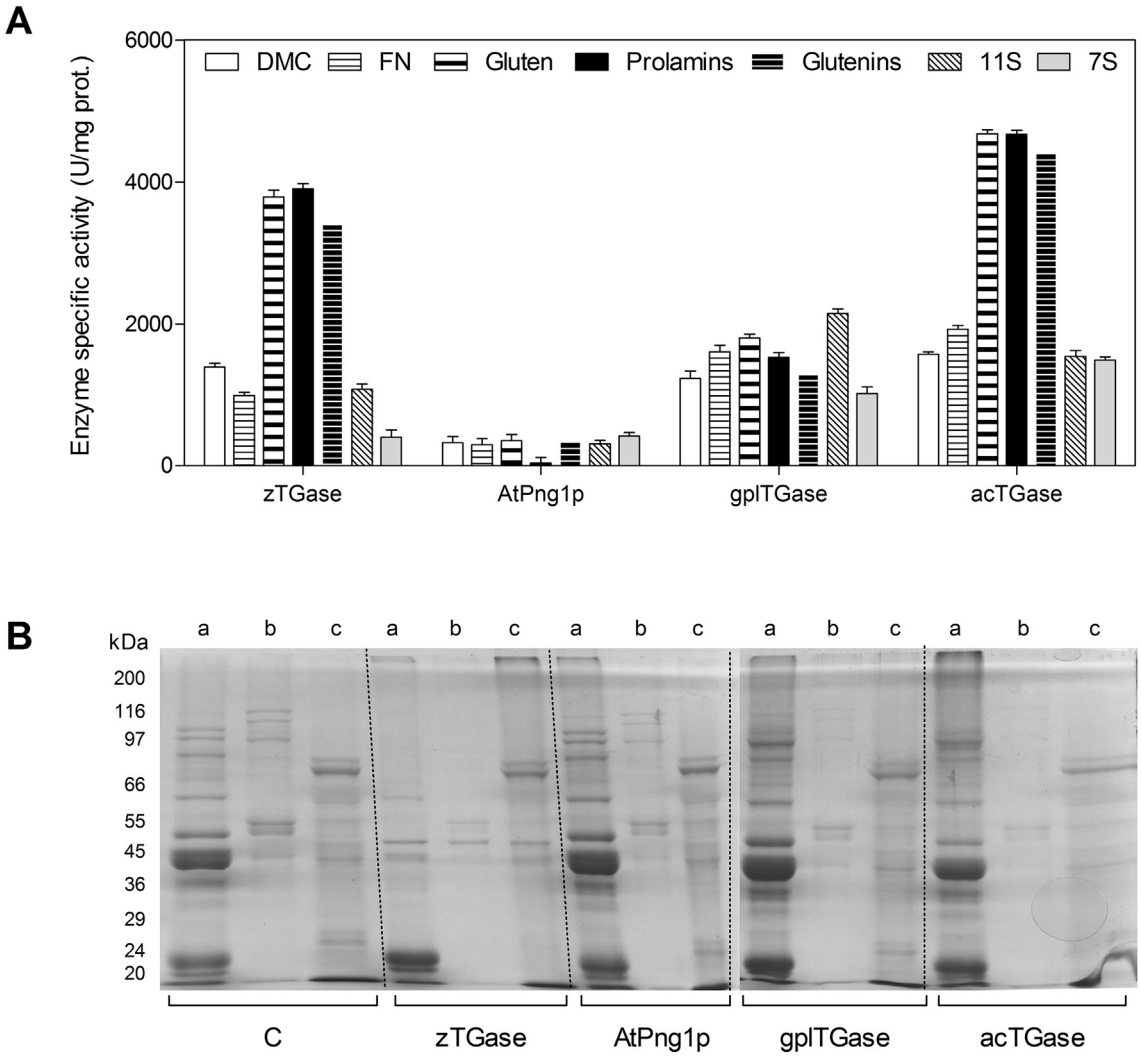

Fig. 1. TGase activity on several protein substrates and consequent formation of HMW products; A) specific enzyme activity, assayed by the microplate biotin cadaverine incorporation method, of mammalian (gplTGase), microbial (acTGase and zTGase), and plant (AtPng1p) TGases; DMC, *N′*, *N′*-dimethyl casein; FN, fibronectin; 7S, vicilin from soy; 11S, legumin from soy. B) Coomassie stained SDS-PAGE showing protein cross-linking effects of TGases on different storage proteins at 30 °C for 90 min of reaction; a 11S, b gluten, c prolamins.

3.3. Combined effects of acTGase and sourdough on proteins

The formation of HMW products, both in Cd and Sd, was ana lysed by separation of the total protein extract before and after acTGase treatment. As shown in Fig. 3A (bracket), HMW products were enhanced by the presence of the sourdough. TGase activity on fractions F1, F2, and F3 extracted from Cd and Sd confirmed that the presence of sourdough significantly increased enzyme activity in a synergistic way as compared with Sd alone, which was not active (Fig. 3 B).

3.4. Physicochemical and rheological features of baked samples

As reported in Table 1, Cb was the softest sample, as it exhibited the lowest hardness value (248 g) as compared with the other samples. With increasing amounts of acTGase, the baked samples reached hardness values of 642 g, 1060 g, and 1177 g with 0.5 U, 1 U and 2 U of enzyme, respectively. Hardness was significantly reduced to values around 500 g when the same units of enzyme were added to Sb. A similar trend in the chewiness parameter was observed as the forces values rose after the addition of acTGase, while in Sb this value remained almost constant even after enzyme addition. Samples made with Sb and acTGase had lower hardness and chewiness values compared to Cb plus enzyme in a dose dependent manner. Cb showed the highest value of cohesiveness and resilience, freshness index, and softness. A reduction of these values was obtained by increasing the enzyme units, while adding the sourdough had no significant effects because it counteracted those induced by the enzyme. Results obtained in the stress relaxation test showed a similar trend to those obtained in the TPA, confirming the synergic benefit of sourdough in combination with acTGase (Table 1).

3.5. Crumb morphological features of baked samples

Bread crumb gas cell size of baked samples were investigated as they have a significant effect on bread texture and on mouth feel perception. As reported in Table 2, similar porosity values, expressed as percentage of hole areas, were observed in all bread slices. All samples were characterized by a fine crumb, i.e., gas cells belonging to classes 1 and 2 in most of the area. The effect of sourdough and acTGase (all concentrations) combined caused an

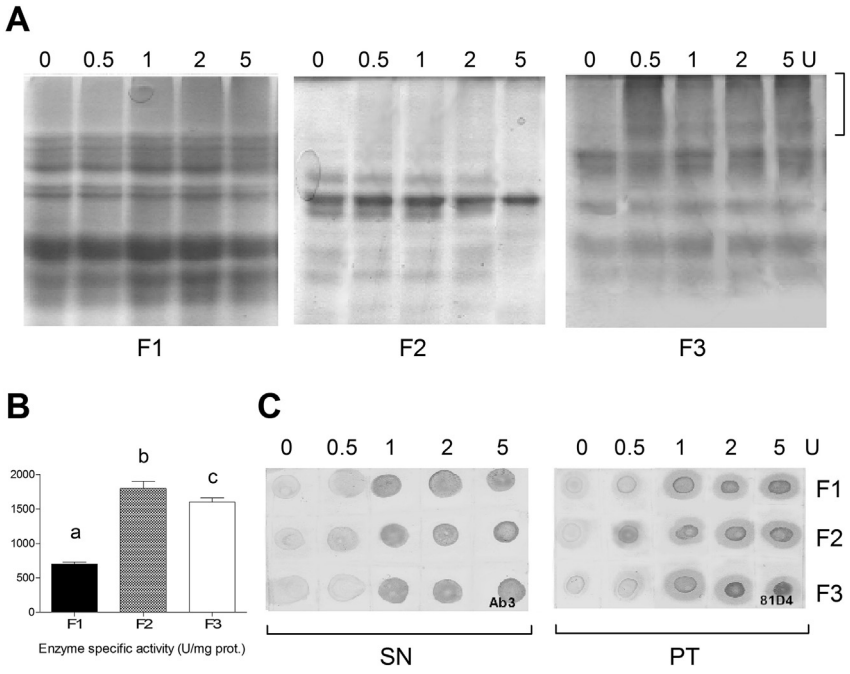

Fig. 2. Dose-dependent cross-linking effects of acTGase on wheat flour protein fractions. A) Electrophoretic patterns of wheat dough protein fractions enriched in albumins/globulins (F1), prolamins (F2), and glutelins (F3), treated with different amounts of acTGase for 90 min; B) specific enzyme activity of acTGase on F1, F2, and F3. C) Dot blot analysis of F1, F2, and F3 treated with different amounts of acTGase and probed with the Ab3 antibody against TGase (supernatant, SN) and 81D4 against cross-links (pellet, PT).

increase in class 2 gas cells but not class 1.

The aspect ratio, an index of product volume, showed that acTGase caused a volume reduction of the slice in a dose dependent manner. The presence of sourdough, in combination with acTGase, increased the volume of the slice, bringing aspect ratio values close to the control sample (Fig. 4). In particular, Cb had the lowest aspect ratio value (0.83 ± 0.04), which increased in a dose dependent manner in samples treated with increasing acTGase units, reaching values of 1.26 ± 0.06. Sb had an aspect ratio of 1.00 ± 0.03, while the addition of 1 U of acTGase slightly decreased this value to 0.96 ± 0.01 (Table 2).

3.6. Combined effect of acTGase and sourdough on volatile compounds

The VC profiles of dough and breads, both Cb and Sb treated or not with acTGase, were determined by GC MS SPME. This approach has proven its potential in providing a VC fingerprint of food and beverages in relation to their microbiota, composition, and/or production processes (Montanari et al., 2016; Patrignani et al., 2016; Scarnato et al., 2016). Sixty molecules belonging to different chemical classes were identified and specific VC finger prints were obtained in relation to the treatment and before and after baking. In particular, Cb was mainly characterized by ethanol, phenylethyl alcohol, and furanones. The addition of TGase and sourdough caused a significant modification of the profile. Since profiles were very complex, data was subjected to PCA. All the samples were mapped in the space spanned by the first two prin cipal components, with PC1 and PC2 explaining 35.48 and 26.00%of the variance, respectively. The score plot showed that samples were clustered mainly according to the baking process and the presence of sourdough (Fig. 5A). Samples were separated into four clusters; cluster 1 grouped Cb samples with and without acTGase, while cluster 2 comprised Sb samples, independent of the enzyme addition. Clusters 3 and 4 included Sd and Cd, respectively. The presence of sourdough separated samples Sd and Sb along PC1 regardless of the presence of the enzyme. The baking process differentiated the samples (Sb and Cb) along PC2. As shown by the loading plots (Fig. 5B), the presence of sourdough strongly affected VC profiles independent of the baking process and enzyme addi tion. In fact, clusters 2 and 3 were characterized by the highest contents of molecules deriving from the activities of sourdough microbiota, including ketones, MCFAs, and alcohols. VCs such as furanone and phenyl ethyl alcohol characterized the baked samples.

3.7. Shelf life of baked products

In order to evaluate the impact of sourdough fermentation on shelf life, the baked samples were monitored during 15 days of storage at RT. A relevant extension (up to 15 days) of the shelf life, in terms of microbial growth prevention (namely of molds and *Bacillus* spp), of bread obtained with sourdough and acTGase addition was observed. After 3 days of storage, the samples did not appear visibly different. After 7 days, Cb was characterized by the presence of molds on the surface. The microbiological analyses, performed at this sampling point, revealed levels of molds and *Bacillus* spp. higher than 5 log CFU/g, that reached about 8 log CFU/g after 15 days of storage. Differently in Sb plus acTGase these species remained under the detection limit after 7 days, while 3 log CFU/g were detected after 15 days of storage (Fig. 6A and B).

4. Discussion

In this work, both the acTGase enzyme and the microbial con sortium consisting of *L. sanfranciscensis* and *C. milleri* alone showed advantages and disadvantages, the latter compensated by the supplementation of the two agents together. The final product showed enrichment in flavor and improved rheological properties. The interaction of microbially produced metabolites or flavor compounds with bread microstructure is generally underestimated and, therefore, not systematically investigated. However, (Aponte

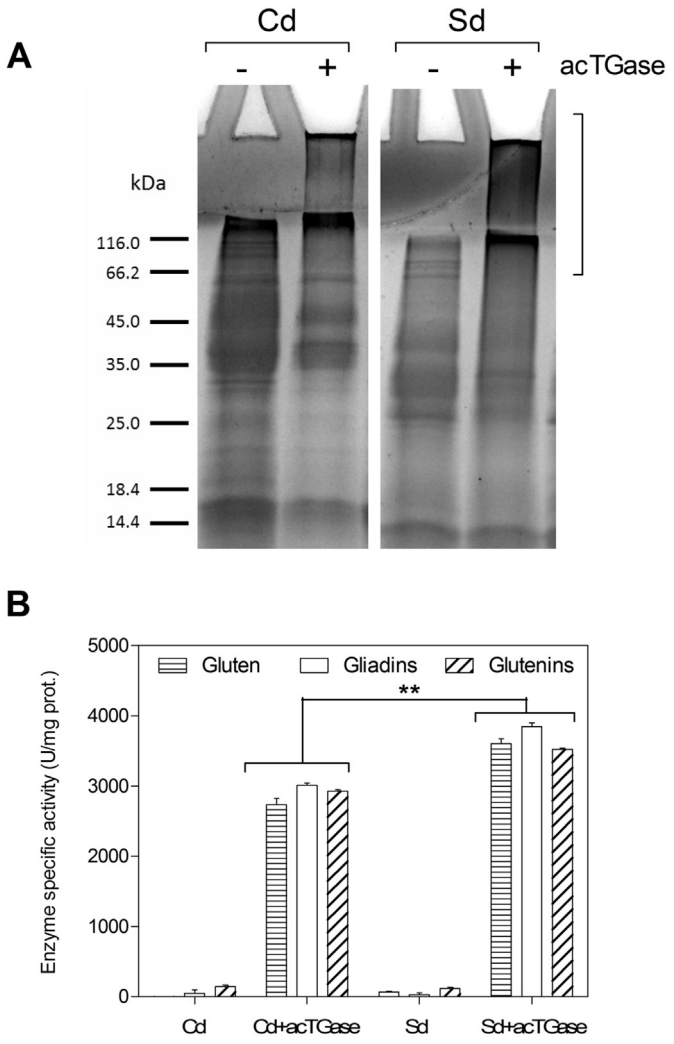

Fig. 3. Combined effects of acTGase and sourdough on wheat dough proteins. A) SDS-PAGE highlighting HMW products (brackets) in total protein extracts from doughs treated with 0.5 U of acTGase and sourdough; Cd conventional dough, Cd + acTGase conventional dough plus acTGase, Sd selected sourdough, Sd + acTGase selected sourdough plus acTGase; B) biotin cadaverine incorporation assay on wheat protein fractions (F1, F2 and F3) in the presence of acTGase and sourdough; Cd conventional dough, Cd + acTGase conventional dough plus acTGase, Sd selected sourdough, Sd + acTGase selected sourdough plus acTGase.

et al., 2014), studying the effect of fermentation time on the microstructure and volatile compounds of chestnut flour based sourdough, showed a significant relationship between the protein network surrounding starch globules and the VC composition of sourdough. Consequently, the specific VC fingerprints obtained here could also be the result of changes induced in the protein network by TGase and/or enzymatic activities of sourdough microflora.

The addition of acTGase led to detrimental effects on Cb. The high hardness and chewiness values of Cb treated with acTGase was, however, counteracted by using sourdough obtained with the selected microbial consortium. These findings are in agreement with the hydrolysis of protein networks that occurs during sour dough fermentation, probably due to a pH mediated activation of cereal proteolytic enzymes (Loponen, Mikola, Katina, Sontag Strohm, & Salovaara, 2004). It is known that LAB may affect prod uct rheology through a strain dependent proteolytic activity (Gobbetti et al., 1996). On the other hand, degradation of wheat gluten would reduce the viscoelastic properties, responsible for the leavening capacity of bread and other baked products.

The main benefits deriving from the use of the microbial con sortium were enriched in flavors, leavening and increased in shelf life. Moreover, it is well known that sourdough contributes to the quality of baked goods as it is rich in nutrients and other com pounds with beneficial health effects (De Vuyst et al., 2014). Under our experimental conditions, sourdough increased the accumula tion of some molecules, such as ketones, MCFAs, and alcohols. These findings are in agreement with evidences underlining that 1 propanol, 3 methyl 2 butanone, 3 hydroxy 2 butanone, and 2 and 3 methyl butanoic acid are the main VCs produced by selected LAB isolated from sourdough (Damiani et al., 1996). Moreover, the interaction between yeast and LAB in sourdough results in a sig nificant enhancement of bread aroma profiles (Ganzle et al., 2007; Hansen & Schieberle, 2005; Kirchhoff & Schieberle, 2001). The proteolytic system of LAB releases low molecular weight peptides and amino acids, which promote the development of metabolic activity in the microorganisms, helping to obtain an improved taste and flavor due to their further production of aroma compounds (Di Cagno et al., 2005; Rizzello et al., 2007). The aroma profile of both dough and baked bread also results from osmotic stress. In fact the exposure of microbial cells to stressful conditions during the fermentation process induces a broad transcriptional response (Serrazanetti, Guerzoni, Corsetti, & Vogel, 2009), reported to affect the organoleptic properties via several metabolic activities (Guerzoni, Vernocchi, Ndagijimana, Gianotti, & Lanciotti, 2007). In particular, it has been reported that *L. sanfranciscensis* in sourdough responds to environmental stresses, with an overproduction of specific compounds such as 3 methyl butanoic acid and 2(5H) furanones (Erasmus, Van Der Merwe, & Van Vuuren, 2003; Guerzoni et al., 2007).

Concerning the effect of TGase on bread shelf life, Gottardi, Khoon Hong, Ndagijimana, and Betti (2014) reported that some

Table 1
Crumb morphological features of baked bread samples analysed by TPA and Relaxation test. Effect of acTGase and sourdough on baked samples rheological properties; Cb convention al bread, Cb + acTGase convention al bread supplied with acTGase, Sb sourdough bread, Sb + TGase sourdough bread supplied with acTGase. Values are means ± standard deviations of three replicates experiments. Means (n 3) followed by the same letter within the same column are not statistically different by using the Kruskal-Wallis in case of significance of the Levene test ($p < 0.05$).

Bread samples	TPA test				Relaxation test	
	Hardness (g)	Cohesiveness	Resilience	Chewiness (g)	Hardness (g)	Springiness (%)
Cb	248 ± 27 b	0.80 ± 0.02 a	0.47 ± 0.01 a	197 ± 18 b	193 ± 27 b	55 ± 0.58 a
Cb + acTGase (0.5 U)	642 ± 39 ab	0.69 ± 0.02 ab	0.38 ± 0.03 ab	439 ± 18 ab	367 ± 28 ab	48 ± 3.08 a
Cb + acTGase (1 U)	1060 ± 95 a	0.67 ± 0.01 ab	0.36 ± 0.01 ab	709 ± 74 a	655 ± 42 ab	49 ± 1.21 a
Cb + acTGase (2 U)	1177 ± 166 a	0.62 ± 0.05 ab	0.28 ± 0.04b	724 ± 61 a	715 ± 52 a	45 ± 5.33 a
Sb	456 ± 5 ab	0.67 ± 0.01 ab	0.36 ± 0.02 ab	307 ± 9 ab	334 ± 23 ab	43 ± 1.69 a
Sb + acTGase (0.5 U)	548 ± 96 ab	0.60 ± 0.04 b	0.31 ± 0.05 ab	331 ± 70 ab	333 ± 44 ab	45 ± 2.61 a
Sb + acTGase (1 U)	368 ± 51 ab	0.71 ± 0.02 ab	0.46 ± 0.02 a	260 ± 32 ab	213 ± 32 ab	45 ± 2.56 a
Sb + acTGase (2 U)	494 ± 43 ab	0.67 ± 0.03 ab	0.40 ± 0.02 ab	330 ± 14 ab	210 ± 21 ab	45 ± 1.44 a

Table 2
Bread crumb gas cell size and aspect ratio. Effect of acTGase and sourdough on crumb morphological features and aspect ratio of baked samples. Holes were identified, counted and classified into 4 predefined area classes on the basis of their size (cm^2): 0.00025 < CLASS 1 < 0.025; 0.025 < CLASS 2 < 0.25; 0.25 < CLASS 3 < 0.5; 0.5 < CLASS 4 < 1; Cb conventional bread, Cb + acTGase conventional bread supplied with acTGase, Sb sourdough bread, Sb + TGase sourdough bread supplied with acTGase. Values are means±standard deviations of three replicates experiments.

Bread samples	Area holes (%)	Area crumb (%)	Classes	Holes (%)	Area (%)	Aspect ratio
Cb	17.55 ± 3.57	82.45 ± 3.57	1	97.81 ± 0.74	50.36 ± 14.45	0.83 ± 0.04
			2	2.02 ± 0.60	30.98 ± 2.85	
			3	0.11 ± 0.10	7.90 ± 5.61	
			4	0.06 ± 0.04	10.76 ± 5.98	
Cb + acTGase (0.5 U)	11.28 ± 1.28	88.72 ± 1.28	1	97.57 ± 0.52	65.81 ± 2.65	0.96 ± 0.03
			2	2.43 ± 0.52	34.19 ± 2.65	
			3	0.00	0.00	
			4	0.00	0.00	
Cb + acTGase (1 U)	12.39 ± 3.07	81.74 ± 0.50	1	98.31 ± 0.07	55.73 ± 1.97	1.26 ± 0.06
			2	1.61 ± 0.08	28.81 ± 1.17	
			3	0.04 ± 0.06	6.62 ± 9.37	
			4	0.03 ± 0.05	8.84 ± 12.50	
Cb + acTGase (2 U)	18.26 ± 0.50	81.74 ± 0.50	1	97.77 ± 0.59	52.94 ± 4.90	1.17 ± 0.02
			2	2.11 ± 0.55	35.24 ± 2.10	
			3	0.06 ± 0.08	4.35 ± 6.15	
			4	0.04 ± 0	7.47 ± 3.35	
Sb	17.18 ± 1.08	82.82 ± 1.08	1	97.08 ± 0.68	57.40 ± 11.72	1.00 ± 0.03
			2	2.92 ± 0.68	42.60 ± 11.72	
			3	0.00	0.00	
			4	0.00	0.00	
Sb + acTGase (0.5 U)	26.79 ± 1.06	73.21 ± 1.06	1	94.66 ± 0.10	24.35 ± 4.78	1.02 ± 0.01
			2	4.70 ± 0.09	40.04 ± 7.31	
			3	0.41 ± 0.01	18.98 ± 4.69	
			4	0.21 ± 0.21	16.63 ± 16.78	
Sb + acTGase (1 U)	18.27 ± 1.16	81.73 ± 1.16	1	93.57 ± 0.55	37.33 ± 4.83	0.96 ± 0.00
			2	6.37 ± 0.47	59.37 ± 0.15	
			3	0.06	3.31 ± 4.68	
			4	0.00	0.00	
Sb + acTGase (2 U)	18.11 ± 1.60	81.89 ± 1.60	1	94.56 ± 0.41	42.33 ± 1.21	0.97 ± 0.01
			2	5.42 ± 0.37	56.41 ± 0.51	
			3	0.03 ± 0.04	1.26 ± 1.78	
			4	0.00	0.00	

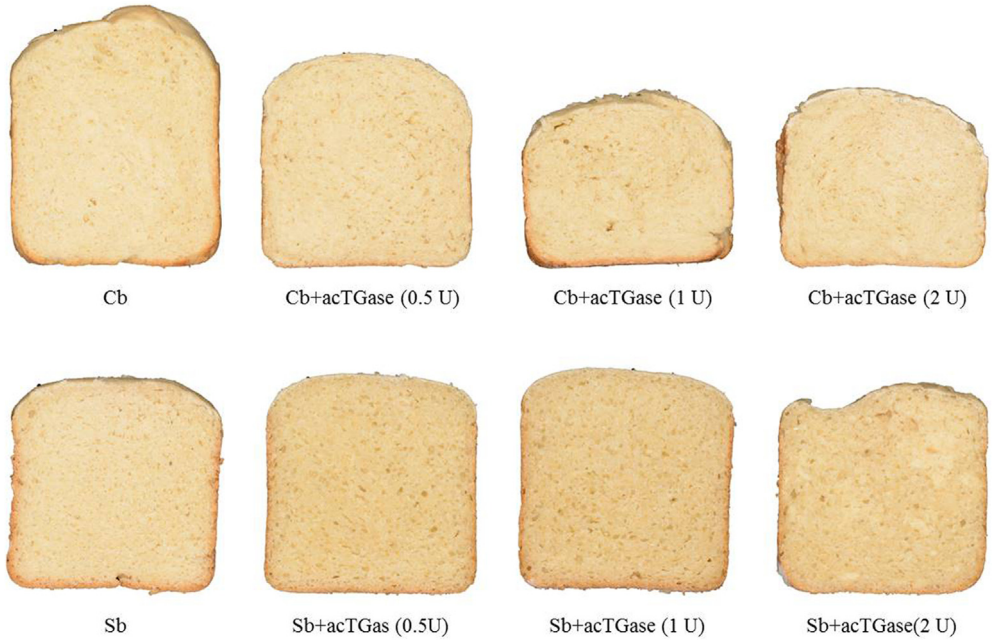

Fig. 4. Representative images of baked sample slices obtained with and without sourdough and different amounts of TGase (0.5, 1 and 2 U/g flour); Cb conventional bread, Cb + acTGase conventional bread supplied with acTGase, Sb sourdough bread, Sb + TGase sourdough bread supplied with acTGase.

peptides from gluten obtained through the activity of TGase showed antimicrobial activity (Gottardi et al., 2014). The release of such peptides can play a role in the modulation of bread microbiota during the storage and consequently on final product shelf life. Caballero et al. (2007) showed an increase of staling when this enzyme is used in gluten based bread. However, Collar and Bollaìn

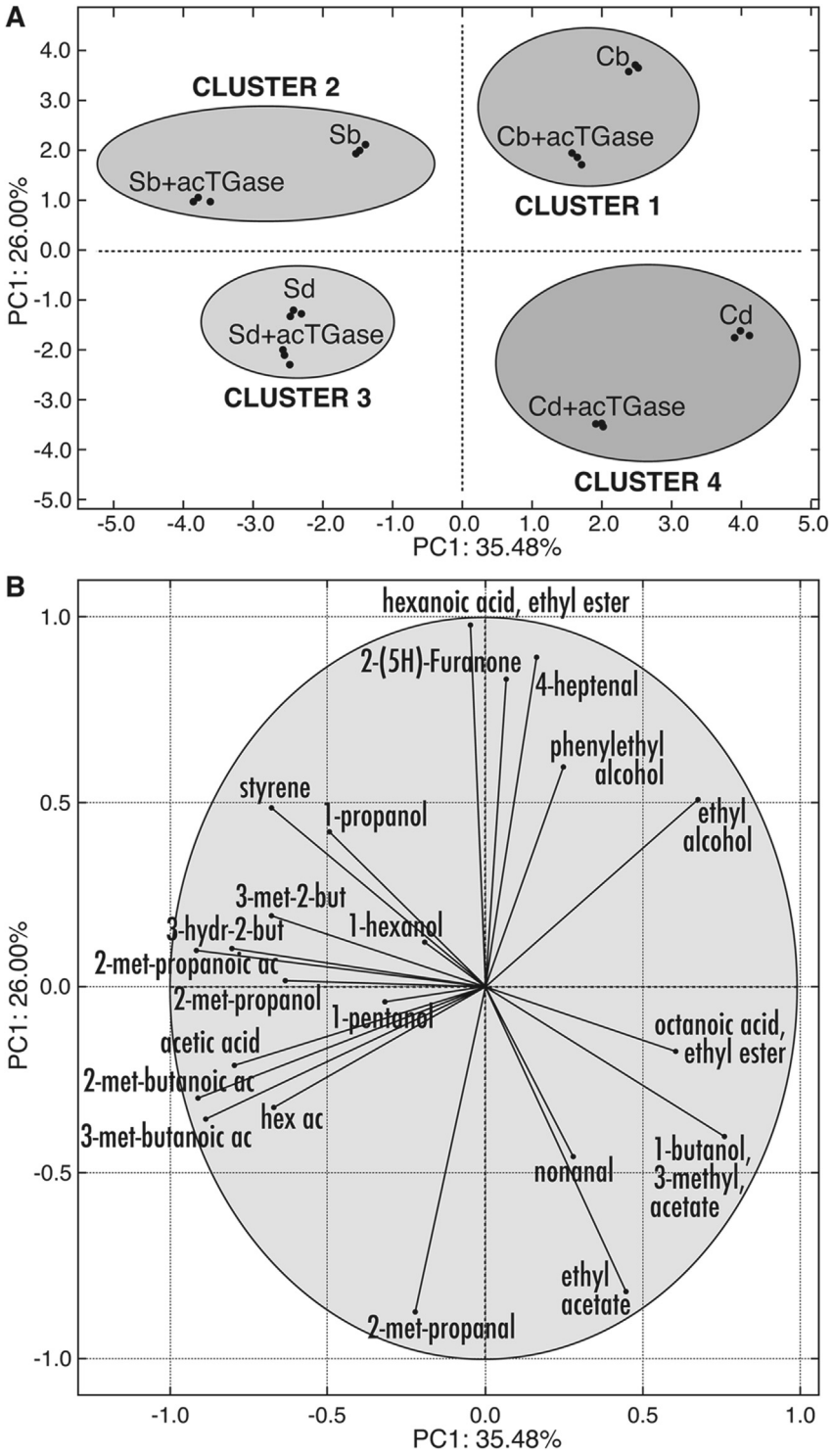

Fig. 5. Principal Component Analysis (PCA) of volatile compounds (VCs) detected in the different doughs (before baking) or breads obtained with or without acTGase (0.5 U/g flour) and with or without sourdough: Cd conventional dough, Cd + TGase conventional dough supplied with acTGase, Cb conventional bread, Cb + acTGase conventional bread supplied with acTGase, Sd selected sourdough, Sd + acTGase selected sourdough supplied with acTGase, Sb sourdough bread, Sb + TGase sourdough bread supplied with acTGase. In particular: A) PCA score plot on the two first factors (PC1 and PC2); B) loadings plots of the VCs obtained from the PCA. Abbreviations: 2-methyl-butanoic acid: 2-met-butanoic ac; 3-methyl-butanoic acid: 3-met-butanoic ac; hexanoic acid: hex ac; 3-methyl-2-butanone: 3-met-2-but; 3-hydroxy-2-butanone: 3-hydr-2-but; 2-methyl-propanoic acid: 2-met-propanoic ac; 2-methyl-propanal: 2-met-propanal.

(2005) reported that the combined use of TGase and α amylase reduced crumb staling kinetics and sensory deterioration (Collar & Bollaìn, 2005). In our experimental conditions, the use of sour dough assured the presence of α amylase released by microbial activity (Sieuwerts, de Bok, Hugenholtz, & van Hylckama Vlieg, 2008), and therefore mitigated the bread staling properties of TGase.

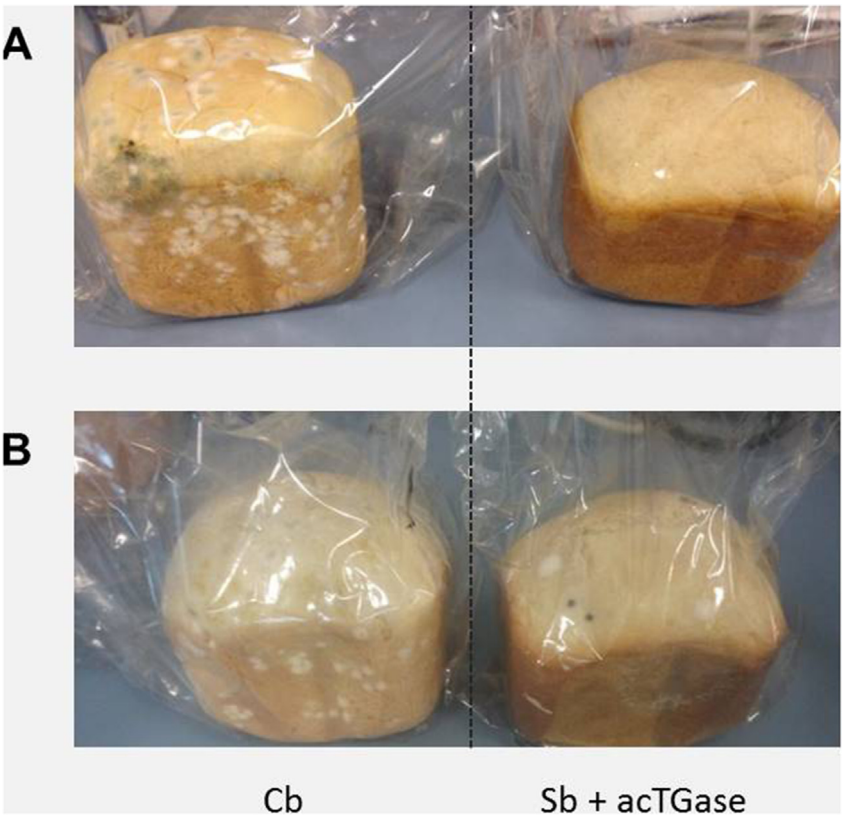

Fig. 6. Shelf-life of baked products obtained using sourdough and 0.5 U/g flour of acTGase (Sb + acTGase), compared to conventional bread (Cb); A) breads after 1 week of storage at RT (24 ± 2 °C); B) breads after 2 weeks of storage at RT.

5. Conclusions

In this work, the synergistic beneficial effects on bread charac teristics of microbial acTGase and a consortium of *L. sanfranciscensis* and *C. milleri* in bread making are described. The excessive hard ness and chewiness of Cb caused by increasing concentrations of acTGase were counteracted by the addition of a proper amount of sourdough. On the other hand, the degradative action of sourdough on protein substrates, which reduces the viscoelastic properties of bread, was compensated by the protein aggregating effect of TGase. In summary, the use of sourdough combined with acTGase showed positive effects on bread rheological features, shelf life, and aroma profile.

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