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Spontaneously fermented ancient wheat sourdoughs in breadmaking: Impact of flour quality on sourdough and bread physico-chemical properties

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ABSTRACT

In this study changes during fermentation of spontaneously fermented modern and ancient (spelt, emmer, and khorasan) wheat sourdoughs and their feasibility to act as leavening agents in breadmaking were evaluated. During 6 h of fermentation, sourdough was characterized for lactic acid bacteria and yeasts counts, pH, total titratable acidity (TTA), proteolytic activity, rheological and electrophoretic profiles. The effects of 25 and 50 g/100g sourdough addition on physico-chemical and sensory properties of refined wheat flour bread were also investigated. Although in all sourdoughs a pH drop, increase in proteolytic activity, gliadin and glutenin degradation with fermentation were recorded, due to different flour composition and microbial activity, tested sourdoughs significantly differed in rheological properties and breadmaking quality. Wheat sourdough being characterized by the highest acidification, increase in proteolytic activity, and the most extensive hydrolysis of gliadins, resulted in bread with the lowest specific volume and hardest crumb texture. Emmer sourdough, having the highest TTA, ash and wet gluten content, exhibited the highest extensibility, the least pronounced changes in proteolytic activity and the electrophoretic pattern which produced bread with the highest volume and softest texture. In general, ancient wheat varieties have shown great potential in sourdough breadmaking in comparison to modern wheat.

1. Introduction

In recent years, ancient wheat varieties have experienced a revival of interest by both consumers and the scientific community. Regarding the nutritional profile, there are some reports which highlighted that the ancient wheat varieties are characterized by a higher content of proteins, soluble dietary fibres, lipids, minerals, vitamins, and bioactive compounds compared to the modern wheat (Angioloni & Collar, 2011; Geisslitz, Longin, Scherf, & Koehler, 2019; Suchowilska, Wiwart, Kandler, & Krska, 2012). In addition to the above, the resurgence of interest in ancient wheat is based primarily on its environment-friendly nature, particularly convenient for low-input and organic farming, which may have an advantage in future agricultural system, especially in terms of biodiversity safeguarding (Boukid, Folloni, Sforza, Vittadini, & Prandi, 2018).

Despite the certain increase in popularity, the exploitation of ancient wheat in the human diet is still untapped. This is mostly governed by their inferior technological quality compared to modern varieties. Even with higher protein and gluten content in ancient wheat flour, the less favourable ratio of gluten subunits causes problems with dough processing and the obtained final product has lower quality (Geisslitz et al., 2019). As the most investigated ancient wheat, spelt gluten is characterized by lower elasticity and higher extensibility compared to modern wheat, yielding weaker and sticky dough, showing difficulty in handling, and bread with lower loaf volume (Frakolaki, Giannou, Topakas, & Tzia, 2018).

Taking into account all these shortcomings, the processing of ancient wheat flour could not rely on the conventional baking process. Based on interviews conducted by Guerrini, Parenti, Angeloni, and Zanoni (2019), sourdough fermentation is one of the most common processes used by artisanal bakeries and this technology has been proposed as a suitable choice for ancient wheat varieties exploitation in breadmaking.

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Although sourdough fermentation has been known to human kind since ancient times, it has attracted the scientific attention in the last 30 years. The sourdough is obtained through a fermentation process of a mixture of flour and water either by native lactic acid bacteria (LAB) and yeasts or by added starters (Menezes et al., 2019). Metabolic activities of specific microbial consortium developed during fermentation ensure a wide range of benefits, such as improving the dough properties, improving and diversifying the sensory quality of bread, and enhancing the shelf life of bakery goods (Chavan & Chavan, 2011). In addition, sourdough is an established technology in stabilising or increasing the levels of various bioactive compounds, retarding starch bioavailability and improving mineral bioavailability (Gobbetti et al., 2019). Moreover, the sourdough technology exhibited undeniable advantages with respect to the standard leavening agents, in terms of sustainability, cultural heritage, and strong consumer interest. However, sourdough technology application on ancient wheat flour is scarcely covered in the relevant literature. Some studies are focusing on the microbiological, rheological, nutritional, and functional characteristics of sourdough/sourdough bread obtained with flour of some ancient wheat species (Coda et al., 2010; Di Renzo, Reale, Boscaino, & Messia, 2018; Shewry et al., 2022; Venturi et al., 2021), but according to our knowledge, they are largely based on fermentation of ancient wheat flour with different isolated species of lactic acid bacteria (type II fermentation). Depending on the strain used as a starter for sourdough fermentation, sourdough bread from ancient wheat species are mostly characterized by lower specific volume, lower crumb firmness and improved mould-free shelf-life in comparison to bread leavened with yeast (Korcari et al., 2021). However, due to the extreme complexity of various phenomena that occur during spontaneous fermentation (type I fermentation), studies dedicated to this type of sourdough fermentation are rare.

Considering that the microflora of sourdough and the features of leavened baked goods are, among others, highly influenced by the nature of the raw materials used, it could be presumed that ancient wheat flours could represent quite original ecological niches for type I sourdough fermentation that will provide bakery products with interesting quality attributes. The study conducted by Coda et al. (2010), which comprised identification of lactic acid bacteria in different wheat varieties, has confirmed the differences in biodiversity between spelt and emmer flour.

In order to investigate the impact of ancient wheat varieties microbial and compositional diversity on their breadmaking potential, the aim of this study was to compare the spontaneously fermented sourdoughs originating from ancient wheat flours with that of modern wheat sourdough. In that manner, the quality of mature sourdoughs, as well as changes that occur during sourdough development in a defined period, were evaluated from chemical, biochemical and rheological aspects. To round out the research, the implementation of spontaneously fermented starters obtained from modern and ancient wheat varieties in wheat flour bread was examined to elucidate their feasibility to act as leavening agents in breadmaking.

2. Material and methods

2.1. Materials

Emmer, spelt, and khorasan grains, representatives of ancient wheat samples (Shewry & Hey, 2015), cultivated at the same location and weather conditions in 2019 harvest year, were obtained from a small local producer (Poljoprivredno gazdinstvo Spelta Jevtić, Bačko Gradište, Serbia), while modern wheat variety, also from the same harvest year, was donated from local milling company Danubius d.o.o. (Novi Sad, Serbia). Before processing, crops were stored for six months, and then hulled crops were dehulled by Heger's large-scale friction de-huller (Herrenberg, Germany). Subsequently, they were milled using a large-scale stone mill Osttiroler Getreidemühlen (Dölsach, Austria). In order to prepare refined wheat flour which will be used as a flour basis in the breadmaking procedure, grains of modern wheat variety were milled using a Bühler laboratory mill (Uzwil, Switzerland).

2.2. Flour characterization

Moisture, ash, starch and total fibre content were determined according to ISO 712:2012, ISO 2171:2012, ISO 10520:1997 and AOAC 985.29, respectively. Protein content was assessed by the Kjeldahl method (ISO 20483:2013), using a nitrogen to protein conversion factor of 5.7. Wet gluten content and gluten index values were determined using ISO 21415–2:2015.

2.3. Sourdough fermentation and analysis

Spontaneously fermented emmer, spelt, khorasan, and modern wheat sourdough was prepared through backslopping procedure (every 24 h, 5 days) in a laboratory incubator (Friocell 111, MMM Medcenter Einrichtungen GmbH, München, Germany). Briefly, flour and demineralized water were mixed in a 1:1 (w/w) ratio with a resulting dough yield [(dough mass/flour mass) × 100] of 200 and incubated at 25 °C for 24 h. After the first fermentation, four additional backslopping steps were carried out by mixing a portion of fermented dough with flour and water (fermented dough:flour:water = 1:2:2 (w/w)) at 24-h intervals. Sourdough prepared in this way was used as a "starter" to initiate the rising process in breadmaking. The starter was combined with the new amount of flour and demineralized water in a 1:2:2 starter:flour:water ratio. Prepared sourdough was kept in a laboratory incubator (Friocell 111, MMM Medcenter Einrichtungen GmbH, München, Germany) at 25 °C for 6 h after which it was used for breadmaking. A period of 6 h was previously established as a time required for making a mature sourdough starter. During this 6 h of fermentation, samples were taken every hour for analysis. For each type of wheat variety (emmer, spelt, khorasan, and modern wheat) a total of 7 samples were collected (0, 1, 2, 3, 4, 5, 6 h) and analysed in duplicate immediately after collection.

2.3.1. Culture media, growth conditions, and enumeration of lactic acid bacteria and yeasts

Microbiological analyses were carried out according to Iacumin et al. (2009), Meroth, Hammes, and Hertel (2003), and Vera, Rigobello, and Demarigny (2009). For lactic acid bacteria (LAB) enumeration, de Man, Rogosa and Sharpe broth (Merck, GmbH, Darmstadt, Germany) supplemented with fructose (0.5 g/100 mL) and maltose (1.0 g/100 mL) (MRS5) and M17 broth (Merck, GmbH, Darmstadt, Germany) supplemented with glucose (0.5 g/100 mL) (GM17) were used. Corresponding agar plates were prepared by adding agar (1.7 g/100 mL, Torlak, Belgrade, Serbia) into each broth. After autoclaving and cooling the medium to 50 °C, 15 mg/L cycloheximide (Sigma-Aldrich, St. Louis, MO, United States) was aseptically added to prevent the growth of yeasts and moulds. For yeasts enumeration YDP medium containing 10 g/L yeast extract (Merck), 20 g/L dextrose (Merck), 20 g/L peptone (Merck), and 17 g/L agar supplemented with 100 mg/L of chloramphenicol (Sigma) was used. One gram of samples collected after 6 h of fermentation was suspended in 9 mL of sterile 0.85 g/100 mL NaCl solution (Sigma), aseptically homogenized in a Stomacher apparatus till consistency. Ten-fold dilutions were performed from 10^{-1} to 10^{-7} and plated on agar plates. Lactic acid bacteria were anaerobically cultured at 30 °C for 48 h on GM17 and anaerobically at 37 °C for 48 h on MRS5. Yeasts were aerobically cultured at 25 °C for 48 h. The obtained results were expressed as colony-forming units (CFU) per gram of sample.

2.3.2. Determination of pH and total titratable acidity

Sourdough samples were subjected to measurement of pH and total titratable acidity (TTA). pH measurements were performed by inserting a solid electrode of a pH meter (HI99161, Hanna Instruments, Padua, Italy) directly into the sourdoughs. TTA of samples collected during 6 h of fermentation was measured after homogenization of dough samples

(10 g) with distilled water (90 g) and expressed as the amount (mL) of 0.1N NaOH to achieve a pH of 8.5.

2.3.3. Proteolytic activity in sourdough samples

The proteolytic activity of sourdough samples was determined as described by Tomić, Torbica, Popović, Hristov, and Nikolovski (2016). The sourdough sample (2.5 g) was suspended in a 7 ml sodium acetate buffer (50 mM, pH 5.0). The reaction was initiated by adding sourdough extract (600 μL) in 2.7 mL of haemoglobin substrate (1 g/100 mL), pH 4.0) and after incubation at 45 °C for 1 h terminated by adding 25 g/100 mL trichloroacetic acid (TCA). TCA-soluble products were determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). At least three replicates were performed for each analysis.

2.3.4. Rheological properties of sourdough

All the sourdough samples collected during 6 h fermentation were subjected to rheological analysis. Rheological measurements were performed in triplicates at 25 ± 0.1 °C using a Haake MARS rheometer (Thermo Scientific, Karlsruhe, Germany). In order to prevent sample slippage, serrated parallel-plate geometry (35 mm diameter) was used and the gap was constant (1 mm) in all rheology tests. After loading a sample, the excess dough was trimmed, covered with paraffin oil to prevent drying during the measurements, and then left to rest for 300 s in order to release residual stress induced during sample loading. Creep was recorded at a shear stress of 50 Pa, for 150 s, followed by a recovery phase of 450 s at a stress of 0 Pa. The monitored parameter was maximum creep compliance (J_{max}).

2.3.5. Protein isolation and SDS-polyacrylamide gel electrophoresis

Emmer, spelt, khorasan, and modern wheat flour as well as freeze-dried sourdough samples (30 mg) after 0, 2, 4, and 6 h of fermentation were extracted with 75 g/100g ethanol (gliadins) and 0.125 M Tris-HCl (pH 6.8) containing 20g/100g glycerol, 10g/100g β-mercaptoethanol, and 4g/100g SDS at 100 °C (glutenins) according to Osborne's classification system (Osborne, 1907) with some modifications. Briefly, supernatant after treatment buffer containing glutenins and supernatants after 70g/100g ethanol containing gliadins were used for SDS-polyacrylamide gel electrophoresis. Gliadins were mixed with sample loading buffer (0.125 mol/L Tris-HCl, pH 6.8, 0.01 mmol/L EDTA, 25g/100g glycerol, 4g/100g SDS, 5g/100g 2-mercaptoethanol, and 0.07g/100g bromophenol blue) at a 1:1 ratio by volume using 4g/100g stacking gel and 12g/100g resolving gel (Vukotić et al., 2015). The destained gels were recorded using ChemiDoc Touch Imaging System with Image Lab Touch Software (Bio-Rad). The intensity of the bands was quantified in ImageJ (National Institutes of Health, NIH) software.

2.4. Breadmaking procedure and bread evaluation

Doughs were prepared by replacing 25 or 50 g/100g of refined wheat flour at 300 g flour basis with previously prepared sourdough samples (wholegrain flour: water content = 1:1) and salt in the amount of 5.1 g (1.7g/100g). Mixing was conducted in a Farinograph mixing bowl (Brabender Technologie GmbH & Co. KG, Germany) and the appropriate amount of water was added in order to achieve final consistency of 400 BU after 5 min of mixing. Additionally, a control sample containing refined wheat flour (270 g), wholegrain wheat flour (30 g), 7.5 g of compressed yeast (2.5g/100g), and 5.1 g of salt (1.7g/100g) was prepared.

After the mixing procedure, dough samples were fermented in a cabinet at 30 °C for 60 min and then punched down. After 30 min of additional fermentation, the amount of 350 g of fermented dough samples were hand-moulded and placed into Teflon pans (L × W × H: 240mm × 85 mm × 65 mm, Tefal, France). Proofing was carried out up to the optimum volume increase (~3 h for sourdough bread and 50 min for control bread) at 30 °C and the relative humidity of 85% for final fermentation. Samples were baked in a modular deck oven (MD, Macpan SNS, Thiene, Italy) at 220 °C until mass loss of 8g/100g. Consequently, bread samples were removed from pans and left to cool down at room temperature for 2 h, sealed in polyethylene bags and stored at 22 °C for further bread quality evaluation. Two batches of each sample were prepared.

2.4.1. Bread volume

Specific bread volume was determined using Volscan Profiler (Stable Micro Systems, Godalming, UK). All tests were carried out on 4 loaves per batch.

2.4.2. Texture measurements

Textural properties of breadcrumb samples were investigated by texture profile analysis (TPA) at room temperature using a TA XT2 Texture Analyser (Stable Micro Systems, Godalming, UK) equipped with a 30-kg load cell and a P/75 (75-mm diameter) aluminium compression platen. All tests were carried out on six slices (35-mm diameter, 10-mm thickness) obtained from the centre of each loaf in a compression mode at pre-test, 1 mm/s; test and post-test speed, 5 mm/s; deformation 75%; and wait time between first and second compression cycles, 5 s, 24 h after baking. The obtained parameters were: hardness, cohesiveness, springiness, chewiness, and resilience.

2.4.3. Colour measurements

Colour parameters of bread crumbs as well as of bread crust were determined in five replicates per bread loaf using a Minolta Chroma Meter CR-400 colorimeter (KonicaMinolta Sensing Inc., Japan) (8 mm Ø contact area), 24 h after baking. The instrument was previously calibrated by a standard light white reference tile and the measurements were carried out under standard illuminant D65. The obtained results were reported according to the CIELab colour system (L^* – lightness, a^* – redness to greenness – positive to negative values, respectively and b^* - yellowness to blueness – positive to negative values, respectively).

2.4.4. Liking study

A total of 84 consumers (39 men and 54 women, aged 23–54 years), regular bread eaters (at least 2–3 days per week), and participants of two days Sourdough bread workshop, were included in the liking study. All participants reported that they are not on a special diet, do not have celiac disease, they are not gluten sensitive or aversive to wheat. The formal assessment was performed on two consecutive days. Participants evaluated ten samples in total, five randomly selected samples each day. A control sample was evaluated on both days. Samples were 10 mm slices from the mid part of the bread, delivered in a monadic way in PET containers with lids, marked with three-digit numbers. Participants evaluated overall liking, appearance liking, odour liking, taste liking and texture liking for each sample on 9-point hedonic scales (1 = I don't like it at all, 9 = I like it very much).

The study was approved by the Ethics Committee of the Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia (Ref. No. 175/I/7–3).

2.5. Statistical analysis

The obtained data were expressed as mean ± standard deviation of individual measurements. Statistical differences between samples were evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's minimum square difference test using Statistica 10.0 (StatSoft Inc., Tulsa, OK, USA), while for sensory data XLSTAT 2022.1.2 was used. The difference between groups was considered significant at the 95% confidence level.

The graphs preparation and statistical analysis for lactic acid bacteria and yeasts count were performed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA).

Table 1
Flour characteristics of modern and ancient wheat varieties.

Sample	Wheat	Spelt	Emmer	Khorasan
Moisture (g/100g)	11.20 ± 0.09^{b}	10.80 ± 0.07^{a}	11.31 ± 0.11^{b}	10.70 ± 0.06^{a}
Ash (g/100 g db)	1.48 ± 0.08^{a}	1.98 ± 0.06^{b}	1.87 ± 0.04^{b}	1.50 ± 0.07^{a}
Total starch (g/100 g db)	72.58 ± 0.34^{c}	67.34 ± 0.34^{a}	71.37 ± 0.32^{b}	70.27 ± 0.35^{b}
Total fibre (g/100 g db)	10.45 ± 0.38^{b}	10.10 ± 0.42^{b}	9.58 ± 0.28^{a}	9.85 ± 0.31^{ab}
Protein (g/100 g db)	11.30 ± 0.32^{a}	15.87 ± 0.37^{b}	15.99 ± 0.30^{b}	11.49 ± 0.43^{a}
Wet gluten (g/100g)	24.7 ± 0.15^{b}	32.2 ± 0.19^{c}	31.0 ± 0.32^{c}	19.2 ± 0.12^{a}
Gluten index (%)	80.0 ± 2.05^{d}	42.0 ± 1.92^{b}	10.0 ± 1.03^{a}	61.0 ± 1.86^{c}

db - dry basis.
The mean values ± standard deviation in the same row are not significantly different ($P > 0.05$) if they are followed by the same letters in the superscript.

3. Results and discussion

3.1. Flour characterization

Modern and ancient wholegrain wheat flours used for sourdough preparation were characterized by their physico-chemical properties, as well as wet gluten content and gluten index as a measure of gluten quality (Table 1).

Among tested varieties, modern wheat was characterized by mostly higher starch and fibre content and lower protein content compared to ancient wheat (spelt, emmer and khorasan) which is in agreement with the results reported by Boukid et al. (2018), Ranhotra, Gelroth, Glaser, and Stallknecht (1996) and Sumczynski, Bubelova, Sneyd, Erb-Weber, and Mlcek (2015). In general, samples differed the most in wet gluten content and gluten index values. Spelt and emmer had the highest wet gluten content, while khorasan was characterized by the lowest amount of wet gluten. Unlike modern wheat, ancient wheat varieties had weak gluten structure which is evident from gluten index values, especially in emmer flour samples.

3.2. Sourdough characterization

3.2.1. Identification of yeasts and LAB in sourdoughs

Yeasts and lactic acid bacteria are microorganisms that possess significant technological characteristics and, hence, play a critical role during sourdough fermentation (Chavan & Chavan, 2011; Coda, Di Cagno, Gobbetti, & Rizzello, 2014). In accordance with that, spontaneously fermented mature sourdoughs obtained from modern and ancient wheat samples were subjected to the determination of the total number of yeasts and lactic acid bacteria (LAB). The total number of yeasts was from 1.4×10^{7} to 6.33×10^{7} CFU/g with a statistically

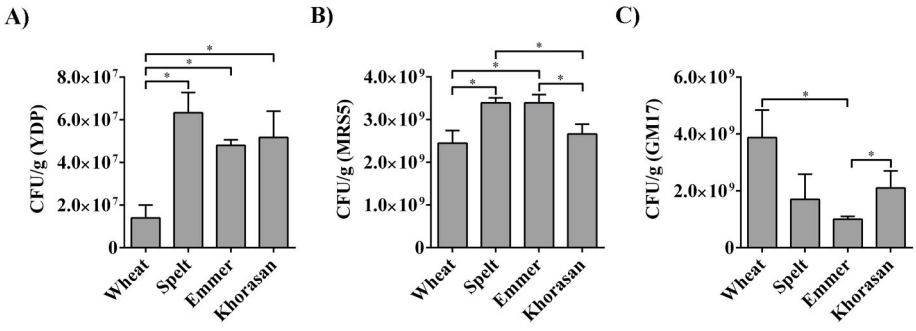

Fig. 1. Cell density (CFU/g) of presumptive yeasts and lactic acid bacteria in modern and ancient wheat sourdough samples: A) Total yeasts on YDP medium, B) *Lactobacillus* on MRS5 and C) *Lactococcus* and *Enterococcus* on GM17. Very extreme points are represented with (*).

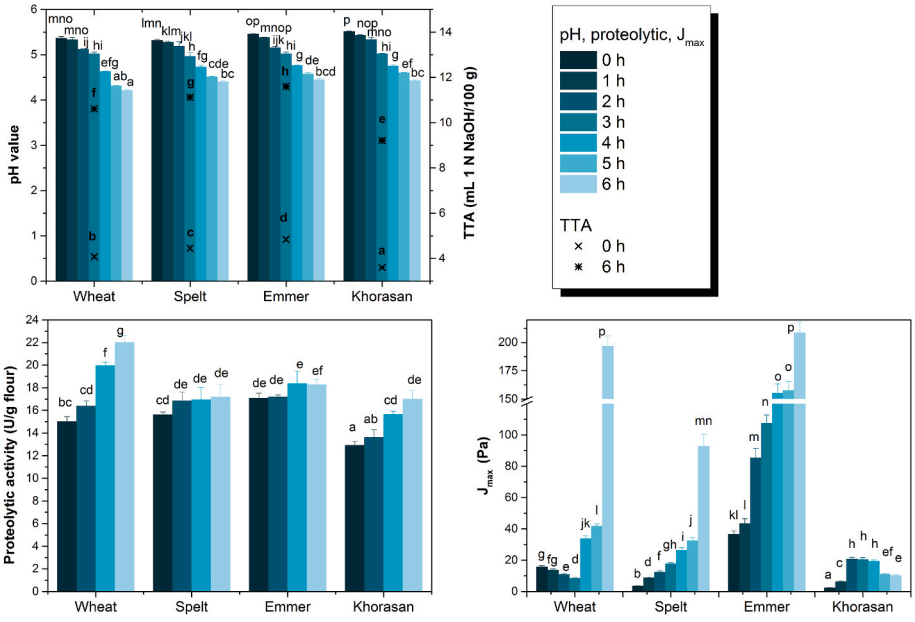

Fig. 2. pH, proteolytic activity, and rheological properties (maximum creep compliance, J_{max}) evolution during wheat, spelt, emmer and khorasan sourdough fermentation presented as histogram (changes from dark to light blue corresponds to fermentation duration from 0 h to 6 h), and total titratable acidity (TTA) of wheat, spelt, emmer and khorasan sourdough at 0 h (x) and 6 h (*) fermentation time. Error bars represent standard deviations of three measurements. Mean values in one histogram with the same lower case letter are not significantly different according to the ANOVA followed by the Tukey's test ($p > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

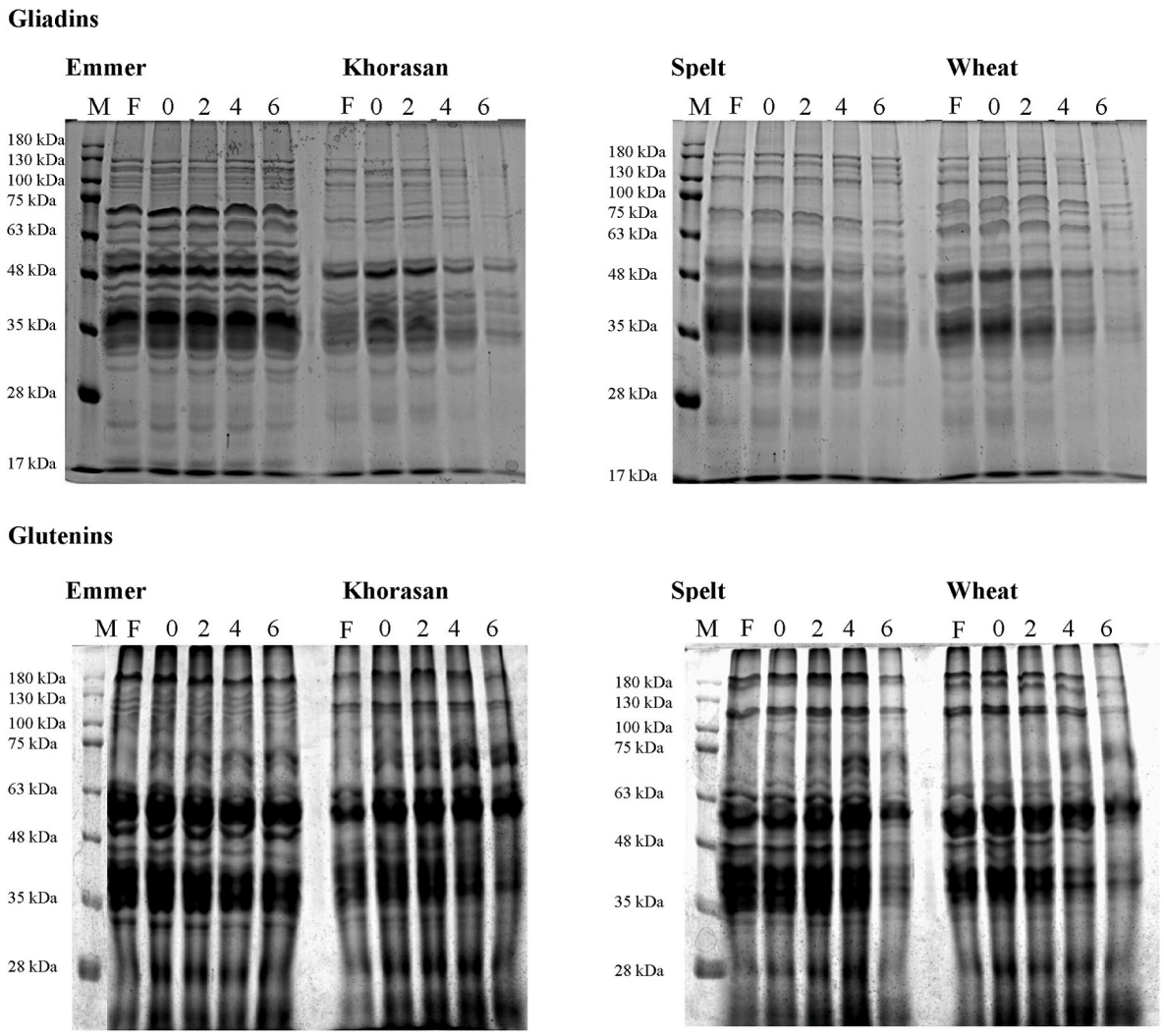

Fig. 3. SDS-PAGE of wheat, spelt, emmer and khorasan flour (F) and sourdough glutenin and gliadin protein fractions after 0, 2, 4 and 6 h of fermentation. M represents a protein marker.

significantly lower number of yeasts in wheat than in the other three spontaneously fermented sourdoughs (Fig. 1A). Further, presumptive LAB enumerated on MRS5 and GM17 ranged from 2.45×10^9 to 3.39×10^9 and from 1.0×10^9 to 3.9×10^9 CFU/g, respectively. Presumptive lactobacilli counted on MRS5 were statistically higher in spelt and emmer than in the other two spontaneously fermented sourdoughs (Fig. 1B). In contrast, the smallest number of presumed lactococci and enterococci was counted in the emmer, while wheat had the highest number of the mentioned bacteria (Fig. 1C). Interestingly, the total number of presumed LAB was cc. 100 times higher than the yeasts in the same spontaneously fermented sourdough. The yeast:LAB ratio of the four sourdoughs is in agreement with previous findings (Lhomme et al., 2015; Liu et al., 2018).

3.2.2. *Changes in pH and TTA during sourdough development*

Mature sourdoughs with specific microbial consortia were used as "starters" to initiate the rising process in breadmaking. The time for whole process was 6 h. This time was previously determined as an optimum time to allow pH drop stabilization, as well as a rise in dough height of a minimum of 100%. When sourdough bread is prepared in households, doubling in initial dough height is considered a final step to incorporating spontaneously fermented sourdough into bread. During these 6 h, sourdough was characterized for pH evolution, while TTA was measured at the initial (0 h) and final point (6 h).

According to results presented in Fig. 2 pH values at 0 h ranged from 5.52 to 5.32, while after 6 h of fermentation there was a drop in pH from 4.45 to 4.22. A stronger pH drop was recorded for wheat and khorasan sourdough than for emmer and spelt. Besides a smaller decline in pH, both emmer and spelt sourdoughs were characterized by higher acidity as supported by TTA values, especially at the initial point (0 h). This can be related to a higher buffering capacity of emmer and spelt samples due to higher protein and mineral content (Hoehnel et al., 2020) as indicated by the ash content presented in Table 1. If factors such as fermentation time and temperature, dough yield and number of back-sloppings are kept constant, pH and TTA values and evolution are impacted by compounds present in the raw material and the complexity of the microbial ecology, i.e. their metabolites (Abedfar & Sadeghi, 2019; Hoehnel et al., 2020). However, considering the higher initial TTA of emmer and spelt samples compared to other wheat samples, it could be assumed that the buffering capacity does rather originate from flour composition than from microbial metabolites.

3.2.3. *Changes in proteolytic activity during sourdough development*

The proteolytic activity in the sourdough system has multiple functions such as providing rapid microbial growth, enhancing the fermentative activity of the sourdough microbiota, and is a key route for obtaining precursor compounds for flavour development during baking (Gänzle, Loponen, & Gobbetti, 2008). The results of proteolytic activity

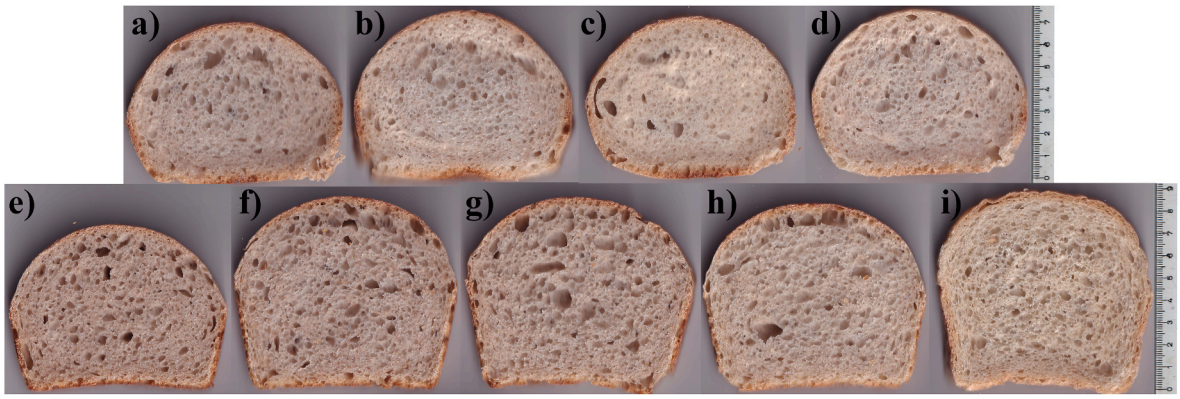

Fig. 4. Impact of wheat variety and sourdough content (25 and 50g/100g) on bread crumb appearance: a) 25g/100g modern wheat, b) 25g/100g spelt, c) 25g/100g emmer, d) 25g/100g khorasan, e) 50g/100g modern wheat, f) 50g/100g spelt, g) 50g/100g emmer, h) 50g/100g khorasan. i) Bread prepared with yeast served as a control sample.

measured during fermentation (Fig. 2) showed that all sourdough systems expressed the same trend of changes. The decrease in pH during fermentation stimulated proteolytic events in all tested samples, but to a different extent. Compared to the initial activity, the most intensive increase was recorded for wheat sourdough where that increase was above 40%. At the end of fermentation, this sample had significantly higher proteolytic activity compared to the sourdough from ancient wheat varieties. The increase in activity with acidification was expected since the aspartic proteinase is the dominating proteinase group of wheat flour with an optimum activity around pH 4.0 (Arendt, Ryan, & Dal Bello, 2007; Loponen, Mikola, Katina, Sontag-Strohm, & Salovaara, 2004). Due to the complexity of a biological system such as sourdough, as well as the difficulty in distinguishing the effects of pH, microbial metabolism, and the activity of indigenous enzymes, the individual contribution of microbial enzymes to overall proteolytic activity remains unclear. Regarding the sourdough with ancient wheat, khorasan sourdough showed the lowest proteolytic activity at the starting point and after 2 h of fermentation, it increased significantly. In the case of sourdough from spelt and emmer, the proteolytic activity did not differ significantly during the entire fermentation period. This is consistent with the fact that these two samples exhibited higher buffering capacity as measured by a moderate decline in pH (Fig. 2) in comparison to wheat and khorasan sourdoughs.

3.2.4. *Changes in the samples' rheology during sourdough development*

The creep test was used to study the effect of wheat variety on the changes in rheological behaviour of sourdough over the course of the fermentation period (Fig. 2). The maximum creep compliance (J_{max}) value, taken from the creep-recovery curve at the end of the creep phase during which constant shear stress is applied, serves as a measure of dough softness. High J_{max} values are indicative of high dough extensibility (Dapčević Hadnađev, Dokić, Hadnađev, Pojić, & Torbica, 2014). In most of the samples, an increase in J_{max} during fermentation was noticed indicating that the dough became significantly softer. Dough softening with fermentation time is in agreement with the findings of Clarke, Schober, Dockery, O'Sullivan, and Arendt (2004) and Kawamura and Yonezawa (1982) who reported that fermentation time influenced the breaking of the large protein aggregates responsible for the dough's structural integrity into small protein aggregates, which contributed to system softening and decrease in elasticity. Protein structure degradation could be attributed to proteolytic activity (Bleukx, Roels, & Delcour, 1997; Gobbetti, Smacchi, Fox, Stepaniak, & Corsetti, 1996; Kawamura & Yonezawa, 1982), and the reduction of disulphide bonds (Kobrehel et al., 1992; Wong et al., 1993). Therefore, dough structure weakening due to protein degradation presented in Fig. 2 is in agreement with increased proteolytic activity over fermentation time detected in this study (Fig. 2). In addition, in an acidic environment, proteins are positive net charged which leads to increased intramolecular electrostatic repulsion forces and consequent unfolding of the gluten proteins which increases their solubility and weakens the structure (Maher Galal, Varriano-Marston, & Johnson, 1978). Therefore, dough softening with fermentation can be partially explained by increased TTA from the initial to final fermentation time (Fig. 2).

However, under the same fermentation conditions, investigated samples exhibited different rheological profiles. Modern wheat flour was characterized by an initial hardening effect followed by pronounced softening as fermentation time prolonged. This could be ascribed to a strong gluten complex (Table 1), which needed some time to absorb water and develop before it was subjected to degradation by cereal and microbial proteases. Electrophoresis results (Fig. 3) have also shown pronounced gliadin and glutenin degradation between 2 h and 4 h of fermentation. On the contrary, khorasan sourdough softened during the first 4 h of fermentation, after which it hardened. Khorasan sourdough was also characterized by decreased extensibility compared to other samples. This could be ascribed to low wet gluten content and increased gluten index (Table 1) which pointed to higher content of elastic over viscous gluten components. With time, viscous glutenin fractions were degraded as evident from Fig. 3, while elastic glutenin subunits remained mostly unchanged which all together contributed to sourdough hardening (Barak, Mudgil, & Khatkar, 2014). In general, during fermentation, emmer sourdough was the most extensible due to the fact that it had the lowest gluten index values (higher contribution of viscous over elastic component) and highest TTA which contributed to protein unfolding and orientation in the direction of applied stress.

3.2.5. *Gliadin and glutenin electrophoretic patterns of sourdough samples*

The electrophoretic patterns which characterize the composition of gliadin and glutenin fractions of tested flours and sourdoughs during fermentation initiated by the natural microbial inoculum are shown in Fig. 3. Differences in the gel band intensities between the native flours and sourdoughs at the initial point of fermentation of both protein fractions were observed, where their intensity was more pronounced in the sourdough samples. This can be explained by the fact that in the circumstances of acidification by lactic acid bacteria and reduction of the disulfide bonds, the solubility of gluten proteins increased, and consequently, the efficiency of extraction was improved (Arendt et al., 2007). Regarding the gliadin fraction, it is obvious that in most sourdough samples, the relative intensities of some of the protein bands were reduced and the disappearance of the bands below 28 kDa was noticeable, which indicates that proteolysis has occurred. These changes were more obvious after 4 h of fermentation. In particular, the hydrolysis of gliadins was more extensive in wheat sourdough which is consistent with the results of proteolytic activity (Fig. 2). On the other hand, sourdough samples made from emmer flour expressed the lowest level of

Table 2
Specific volume, texture and colour parameters of bread containing 25 and 50g/100g sourdough.

Bread sample	Spec. vol. (mL/g)	Crumb texture					Crumb colour			Crust colour		
		Hardness (N)	Springiness	Cohesiveness	Chewiness (N)	Resilience	*L**	*a**	*b**	*L**	*a**	*b**
25g/100g wheat	2.07 ± 0.03^{b}	213.5 ± 18.2^{c}	0.947 ± 0.008^{ab}	0.596 ± 0.008^{a}	120.1 ± 10.1^{c}	0.349 ± 0.11^{d}	66.47 ± 1.22^{a}	1.79 ± 0.16^{c}	16.99 ± 0.39^{ab}	51.05 ± 0.95^{ab}	11.67 ± 0.38^{c}	25.40 ± 0.61^{a}
25/100g spelt	2.03 ± 0.09^{ab}	265.4 ± 9.1^{d}	0.935 ± 0.012^{a}	0.594 ± 0.006^{a}	147.5 ± 8.3^{d}	0.351 ± 0.012^{d}	70.42 ± 1.04^{cd}	0.60 ± 0.25^{a}	18.20 ± 0.41^{c}	54.50 ± 1.08^{bc}	11.28 ± 0.38^{bc}	28.12 ± 0.27^{abc}
25g/100g emmer	1.89 ± 0.04^{a}	250.7 ± 13.3^{d}	0.933 ± 0.018^{a}	0.604 ± 0.009^{ab}	140.6 ± 11.2^{d}	0.357 ± 0.016^{d}	67.68 ± 1.42^{ab}	1.19 ± 0.15^{b}	17.38 ± 0.51^{abc}	56.96 ± 1.79^{c}	10.38 ± 0.96^{bc}	26.56 ± 1.05^{ab}
25/100g khorasan	1.99 ± 0.05^{ab}	246.5 ± 23.7^{d}	0.948 ± 0.011^{ab}	0.609 ± 0.008^{b}	142.1 ± 11.4^{d}	0.360 ± 0.009^{d}	69.69 ± 1.30^{bc}	0.60 ± 0.19^{a}	17.71 ± 0.35^{abc}	63.10 ± 0.85^{d}	6.12 ± 0.50^{a}	26.27 ± 0.38^{ab}
50g/100g wheat	2.26 ± 0.04^{c}	139.6 ± 12.9^{b}	0.928 ± 0.011^{a}	0.601 ± 0.003^{ab}	77.9 ± 7.7^{b}	0.294 ± 0.006^{b}	66.32 ± 1.67^{a}	3.33 ± 0.31^{e}	17.93 ± 0.83^{bc}	49.66 ± 3.55^{a}	13.84 ± 1.59^{d}	28.67 ± 1.76^{bcd}
50/100g spelt	2.80 ± 0.01^{d}	73.6 ± 3.5^{a}	0.945 ± 0.003^{ab}	0.629 ± 0.010^{c}	43.4 ± 2.5^{a}	0.285 ± 0.006^{ab}	72.40 ± 0.87^{de}	2.48 ± 0.28^{d}	16.75 ± 0.69^{a}	50.09 ± 1.57^{ab}	14.51 ± 0.85^{d}	29.83 ± 1.37^{cd}
50g/100g emmer	2.84 ± 0.02^{d}	54.8 ± 2.3^{a}	0.941 ± 0.008^{ab}	0.631 ± 0.005^{c}	32.9 ± 1.4^{a}	0.269 ± 0.006^{a}	73.1 ± 0.99^{ef}	2.15 ± 0.17^{cd}	17.49 ± 0.32^{abc}	46.71 ± 3.19^{a}	15.53 ± 1.35^{d}	28.51 ± 1.98^{bcd}
50/100g khorasan	2.67 ± 0.09^{d}	75.9 ± 8.6^{a}	0.957 ± 0.007^{b}	0.650 ± 0.004^{d}	46.8 ± 4.7^{a}	0.323 ± 0.006^{c}	75.5 ± 2.17^{f}	1.19 ± 0.35^{b}	17.29 ± 0.74^{abc}	49.39 ± 2.50^{a}	15.71 ± 1.12^{d}	31.02 ± 1.72^{d}
control	2.77 ± 0.06^{d}	120.0 ± 6.6^{b}	0.959 ± 0.007^{b}	0.593 ± 0.004^{a}	68.3 ± 4.2^{b}	0.304 ± 0.005^{b}	69.13 ± 0.78^{bc}	0.93 ± 0.32^{ab}	19.25 ± 0.67^{d}	61.43 ± 1.23^{d}	9.30 ± 0.35^{b}	28.01 ± 0.59^{abc}

The mean values ± standard deviation in the same column are not significantly different ($P > 0.05$) if they are followed by the same letters in the superscript.

changes. Besides the slight decrease in band intensities, as fermentation proceeded, a fainter protein band between 28 and 35 kDa appeared. Glutenin extracts of all tested sourdough samples showed the same band gel profile as native flours. The changes in glutenin were mainly detectable in sourdough samples after 6-h of fermentation with the most intense changes being observed in wheat and spelt sourdoughs. In these samples, protein patterns were characterized by decreasing intensity of bands with molecular mass above 75 kDa and in the range of 35–48 kDa. Overall, the SDS-PAGE protein patterns showed that the degradation of gliadins was more extensive when compared to the glutenin fraction.

3.3. Sourdough bread characterization

3.3.1. Bread volume and texture

In this study, bread prepared with 50g/100g sourdough exhibited higher specific loaf volumes, comparable to the one of yeast-fermented control bread, than bread with 25g/100g sourdough addition (Fig. 4; Table 2). Since loaf-specific volume is mainly affected by the magnitude of gas that is produced over fermentation and the gas-retaining capacity of the dough (Abedfar & Sadeghi, 2019), it can be concluded that 25g/100g sourdough addition was not enough to produce high quantities of gases during proofing. However, 50g/100g emmer, spelt and khorasan sourdough addition was enough to increase gas production during fermentation and led to balanced macromolecular degradation that can contribute to the gas-retaining capacity of the dough and consequent higher loaf volumes in comparison to the yeast-fermented dough (Table 2). In addition, for all sourdough containing bread crumbs, a few number of large pores could be noticed (Fig. 4) which was not the characteristic of control bread. Results of the study performed by Dal Bello et al. (2007) have shown that sourdough improves the gas holding capacity and the development characteristics of the dough when compared to chemically acidified or non-acidified yeast fermented doughs. The effect of sourdough addition on bread specific volume increase was also confirmed by previous researchers (Axel et al., 2015; Olojede, Sanni, & Banwo, 2020). On the contrary, 50g/100g wheat sourdough produced loaves with lower specific volume compared to control bread and bread containing 50g/100g sourdough from ancient wheat varieties. This could be supported by the results of electrophoretic measurements (Fig. 3) according to which sourdough based on wheat flour was characterized by the highest degradation of gliadin and glutenin fractions thus leading to a system with a lack of structure-forming and gas-holding agents during breadmaking and consequently lower loaf volume.

Texture analysis (Table 2) revealed that the addition of 25g/100g of sourdough resulted in a bread with increased hardness compared to control bread. The obtained findings could be related to lower specific volume values and consequently denser structure in comparison to the control sample. However, the addition of 50g/100g emmer, khorasan and spelt sourdough influenced the softer crumb texture, while the wheat flour-based sourdough resulted in a slight, but insignificant increase in hardness values compared to the control sample. Bread prepared with emmer sourdough was characterized by the highest specific volume value and also had the lowest hardness values; whereas bread containing 50g/100g wheat-flour based sourdough that was characterized by the lowest specific volume values had the highest hardness values at 50g/100g flour replacement level.

A similar trend was observed for the chewiness parameter, which is defined as the energy required to masticate a solid food product (Ding, Peng, Li, & Yang, 2019). Concerning the cohesiveness parameter (the ratio obtained under the second peak curve to the area of the first peak), it can be concluded that sourdough addition resulted in a cohesiveness increase which was more pronounced at the 50g/100g replacement level in comparison to 25g/100g replacement level. Generally, the addition of emmer, khorasan and spelt sourdough at a 50g/100g replacement level resulted in a bread hardness and chewiness decrease as well cohesiveness increase in comparison to the control sample.

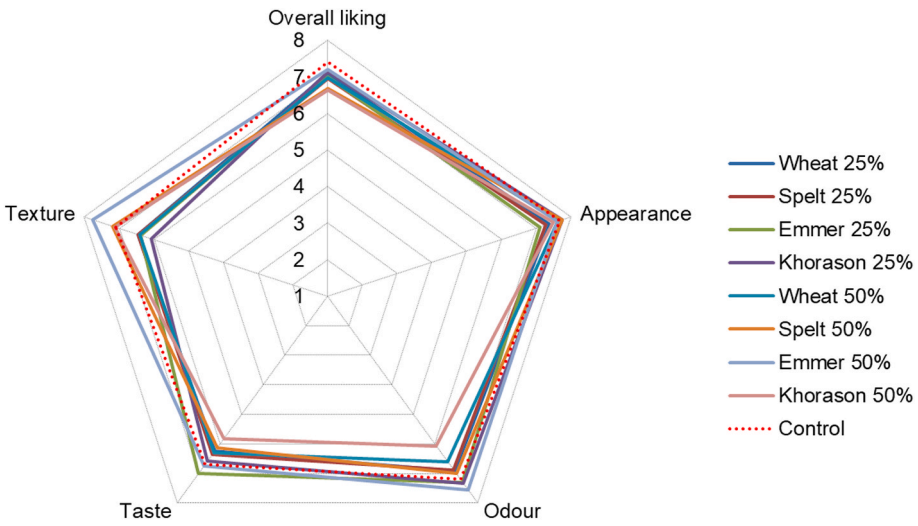

Fig. 5. The liking study of bread samples containing 25 and 50g/100g of wheat, spelt, emmer and khorasan sourdough compared to yeast-fermented bread which served as a control.

Similar behaviour was noted by Jitrakbumrung and Therdthai (2014) and Jung, Park, and Lee (2017) who revealed that sourdough addition resulted in a bread hardness and chewiness decrease as well cohesiveness increase.

3.3.2. Bread crumb and crust colour

The addition of 50g/100g emmer, khorasan and spelt sourdough resulted in higher bread crumb L^* values compared to the control sample, while 50g/100g wheat sourdough bread had a lower L^* value (Table 2). The obtained findings could be related to bread specific volume. Namely, samples which were characterized by higher specific volume had lighter bread crumbs than control bread.

However, lower values of bread crust L^* parameter for the majority of sourdough-containing bread samples in comparison to control bread was observed. Moreover, an increase in sourdough content led to darker crust formation which is in agreement with Torrieri, Pepe, Ventorino, Masi, and Cavella (2014). A similar trend can be seen for a^* parameter. In general, during sourdough preparation, an increase in proteolytic activity was observed (Fig. 2) leading to higher amounts of free amino acid lysine available for Maillard reaction with reducing sugars. Moreover, according to Gänzle (2014), sourdough fermentation of wheat and rye increases amylase activity which results in maltodextrins, maltose and glucose liberation thus causing the increased browning reaction during the baking process. In general, besides physico-chemical characteristics of the raw material (i.e. dough moisture content, pH, reducing sugars and amino acid content), crust colour is also influenced by the processing conditions applied during baking such as temperature, relative humidity, oven type, etc. (Torrieri et al., 2014).

3.3.3. Liking study

The results of the liking study are presented in Fig. 5. Bread samples were considered acceptable if their mean scores for overall liking were above 5 (neither like nor dislike). Although slight differences in the degree of liking between bread samples were perceived, the statistical analysis showed that there were no statistical differences ($p < 0.05$) in the mean values neither for overall liking nor liking of appearance, taste, odour or texture, and all mean values were above the defined threshold of 5.0. Overall liking of bread samples was in the range of 6.63 for 50g/100g khorasan bread to 7.41 for control bread, showing that samples were classified as slightly or moderately liked. The lower liking of the khorasan sample is probably due to the slightly lower liking of taste and odour of this sample (liking of taste 5.81, liking of odour 6.06). In general, since sourdough bread is not common in the Serbian market where consumers are more familiar with yeast-fermented bread, the obtained results were expected. Škrobot et al. (2022) have also revealed that consumers strongly penalized buckwheat pasta samples compared to the control one due to the inappropriate taste and flavour of the former.

4. Conclusions

Results of this study have shown that the breadmaking quality of ancient wheat varieties such as spelt, emmer and khorasan, which do not perform well in conventional baking tests, can be improved using sourdough technology. Due to different flour compositions and microbial consortia formed during spontaneous fermentation, ancient wheat varieties responded differently than modern wheat in terms of buffering capacity, proteolytic activity, glutenin and gliadin hydrolysis pattern and dough rheological behaviour. Therefore, spontaneously fermented ancient wheat samples produced bread of specific volume and crumb texture comparable to the one of yeast-fermented bread and superior to these observed for modern wheat sourdough bread. Although bread containing yeast was the most preferred among consumers participating in the liking study, ancient wheats can be promising raw materials in sourdough breadmaking taking into account that sourdough bread popularity has increased during a covid-19 pandemic, which could influence changes in consumers' preference in favour of sourdough bread taste and aroma.

CRediT authorship contribution statement

Jelena Tomić: Writing – original draft, preparation, Methodology, Supervision. **Tamara Dapčević-Hadnađev:** Conceptualization, Writing – original draft, preparation. **Dubravka Škrobot:** Formal analysis, Visualization, Data curation. **Nikola Maravić:** Investigation. **Nikola Popović:** Supervision, Validation, Resources. **Dušan Stevanović:** Investigation. **Miroslav Hadnađev:** Writing – review & editing, Resources, Project administration, Funding acquisition.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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