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Protein fortification of wheat bread using dry fractionated chickpea protein-enriched fraction or its sourdough

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ABSTRACT

Chickpea protein-enriched ingredients were prepared by combining dry milling, air classification, and optionally solid-state fermentation. The fermentation was carried out with the autochthonous LAB strain *Pediococcus acidilactici* to reduce the level of antinutritional factors. A protein-enriched chickpea fraction and its sourdough were used to partially replace wheat flour with 20%–30% w/w in wheat bread. The protein content of bread increased by 38.5% on dry basis with a 30% w/w replacement. As the substitution level increased from 0% to 20% and 30%, a longer dough mixing time was required, the specific volume of the bread decreased, and the crumb structure became denser. The levels of raffinose, stachyose, and verbascose in the sourdough bread were reduced by 75.4%, 97.6%, and 90.0% compared to the unfermented bread, respectively. With sourdough addition the crust showed less browning and exhibited a better microbiological stability compared to that of the other breads.

1. Introduction

The growing world population and increasing prosperity demand an increasing supply of dietary protein (Schutyser, Pelgrom, Van der Goot, & Boom, 2015). The use of more plant protein in our diet will reduce the use of primary agricultural resources and lead to lower greenhouse gas emissions compared to animal protein (Aiking, 2014; Mattila et al., 2018). In this respect, legumes are an excellent source of dietary protein. The protein content of pulses like pea, bean, chickpea, lupine, and lentil is between 17 and 46%, while cereals such as wheat, maize, and sorghum only have a protein content of 8–13% (Foschia, Horstmann, Arendt, & Zannini, 2017; Nkhabutlane, du Rand, & de Kock, 2014). Moreover, regular consumption of pulses has been advised to mitigate the risk of cardiovascular diseases, diabetes, and high serum cholesterol (Asif, Rooney, Ali, & Riaz, 2013; Sokolowski, Higgins, Vishwanathan, & Evans, 2019). Bakery products made with wheat are amongst the most consumed staple foods for many ethnic groups. The incorporation of pulse flours in wheat bread can produce protein-enriched bread with increased nutritional value thanks to the well-balanced amino acid profile and high fibre contents of pulses (Boukid, Zannini, Carini, & Vittadini, 2019).

Chickpeas have been applied in bakery products for its moderate calories, high protein (17–22%), complex carbohydrates, dietary fibres, vitamin, and less beany flavor (Asif et al., 2013). Previous studies reported that wheat dough that was fortified with up to 10% chickpea flour still had a non-sticky surface and yielded a bread crust with similar colour as plain wheat bread (Boukid et al., 2019). However, higher levels of replacement lead to decreased dough stability and resistance, resulting in a stiffer bread with smaller volume. These effects may be explained by the dilution of the gluten content and interactions among fiber components, water and gluten. Overall, studies show that breads can only be reasonably prepared with low levels of replacement, even though fermentation and bread improvers (e.g. xanthan gum) could somewhat mitigate the effects (Shrivastava & Chakraborty, 2018). To further increase the chickpea protein level in bread without impacting the quality too much, some studies reported the addition of wet-isolated protein (Boukid et al., 2019). However, the wet isolation of proteins from legumes is resource intensive and creates significant waste streams.

In this study, we therefore propose the incorporation of protein-enriched chickpea fractions obtained via dry fractionation, which involves the combination of dry milling and air classification. Dry fractionation requires much less energy, produces less waste and retains the native protein functionality compared to conventional wet extraction methods (Xing et al., 2020). However, the presence of anti-nutritional factors (ANFs) such as flatulence-causing oligosaccharides (raffinose, stachyose, and verbascose), phytic acids, and trypsin inhibitors in

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air-classified raw protein fractions is less desired for food application (Khattab & Arntfield, 2009). Therefore, solid-state fermentation (SSF) is used as a cost-effective approach to eliminate these ANFs in the chickpea fraction (Shrivastava et al., 2018). In our previous study, autochthonous lactic acids bacteria (LAB) presented in chickpea protein-enriched (fine) fractions were identified. A strain that belongs to the *Pediococcus acidilactici* was selected as starter culture based on its ability to selectively metabolize raffinose oligosaccharides (Xing et al., 2020).

A dry-enriched chickpea protein-enriched (fine) fraction was first produced by milling and air-classification. Subsequently, an autochthonous *P. acidilactici* strain was inoculated to that fraction which was then fermented in solid-state to obtain chickpea sourdough. Finally, wheat flour was partially replaced by protein-enriched chickpea or its sourdough during bread making, and the quality of the obtained breads was evaluated on properties in terms of protein content, ANFs content, colour, specific volume, texture, and microbiological stability, etc.

2. Materials and methods

2.1. Materials

Chickpea (Cicer arietinum) seeds (Kabuli) were purchased from a retailer (Biologische Toko, The Netherlands). The protein, carbohydrate, fat, and ash contents were 20.6 g/100 g, 70.2 g/100 g, 6.0 g/100 g, and 3.2 g/100 g on dry basis, respectively. The seeds were stored in a tightly screwed polyethylene container at 4 °C. Wheat flour (Jumbo, The Netherlands), salt (JOZO, The Netherlands), and yeast (Dr. Oetker, The Netherlands) were bought from a local supermarket. The protein, carbohydrate, fat, and ash contents of the wheat flour were 11.7 g/100 g, 85.4 g/100 g, 0.8 g/100 g, and 2.1 g/100 g on dry basis, respectively. The flour was stored in a dry cabinet at room temperature.

2.2. Preparation of chickpea protein-enriched (fine) fraction

Whole chickpea seeds were coarsely ground into chickpea grits with a pin mill (LV 15 M, Condux-Werk, Germany). The chickpea grits were then milled into chickpea flour with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) at ambient temperature. The feed rate was 0.5 kg/h, the air flow rate was 40 m^3/h, and the air classifier wheel speed was fixed at 2900 rpm (Xing et al., 2020). A chickpea protein-enriched (fine) fraction was obtained by air classification with an ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany) at ambient temperature. The feed rate was 0.2 kg/h, the air flow rate was 52 m^3/h, and the speed for the air classifier wheel was adjusted to 10,000 rpm (Xing et al., 2020).

2.3. Definition of bread formulation

The bread was prepared by the straight dough method (de Oliveira, da Silva Lucas, Cadaval, & Mellado, 2017). Wheat bread with the recipe that is reported in Table 1 was used as a control. The chickpea protein-enriched fraction was blended with wheat flour in different ratios (20:80 and 30:70). The addition of water was determined for each formulation with a farinograph (Brabender GmbH & Co. KG, Duisburg, Germany) based on the AACC method 54–21. For each measurement, 50 g of mixed flour was loaded in the farinograph mixer chamber. With continuous mixing at a speed of 63 rpm for 0.5 min, a certain amount of water was added. A water bath was connected to keep the farinograph at 30 °C during measurement (Zhang et al., 2019). Each farinograph experiment was recorded using WINMIX software for 15 min. The water absorption (the amount of water taken up by flour to yield the desired dough resistance), the dough development time (the time required from the moment the water is added to the maximum torque value is reached), and the dough stability (the time that dough consistency is kept at 0.98 Nm/500 Brabender Units) of different formulations were determined. Two breads were produced for each bread trial. Duplicate measurements were performed for each formulation.

Table 1
Formulations of wheat bread (control) and bread fortified with chickpea protein-enriched fraction or its sourdough at substitution levels of 20% and 30%.

Ingredients	Wheat bread (control)	Bread fortified with chickpea protein-enriched fraction		Bread fortified with sourdough	
		20%	30%	20%	30%
Wheat flour (g)	60.2	50.0	45.7	50.0	45.7
Chickpea protein-enriched fraction (g)	–	12.5	18.7	–	–
Sourdough (g)	–	–	–	18.8	29.4
Water (g)	37.3	35.0	32.2	28.7	22.4
Yeast (g)	1.5	1.5	1.5	1.5	1.5
Salt (g)	1.0	1.0	1.0	1.0	1.0

2.4. Preparation of chickpea sourdough

Based on previous work, the *Pediococcus acidilactici* strain AA106 (Accession number: KY940561) was selected to ferment chickpea protein-enriched fraction (Xing et al., 2020). To be specific, a loop of bacteria taken from a frozen stock (−80 °C) was inoculated into a 10 ml De Man, Rogosa and Sharpe (MRS) broth (Merck, Germany) tube and incubated for 24 h at 30 °C. One milliliter of MRS broth cell suspension was transferred to a new 9 ml MRS broth tube and incubated at 30 °C for another 24 h. The cells were then harvested by centrifuging 1 ml MRS broth cell suspension at 10,000 rpm for 5 min. After discarding the supernatant, the pellet was re-suspended in 1 ml peptone physiological salt solution (PPS) (Tritium Microbiologie B·V., The Netherlands) to wash the cells. The centrifugation step was repeated, and the starter culture was prepared by suspending the cell pellet in 1 ml PPS. For 100 g of chickpea protein-enriched fraction, 50 ml of Milli-Q water and 0.5 ml of starter culture solution was added and well mixed manually. The dough was anaerobically incubated at 37 °C for 72 h.

2.5. Bread making

The wheat flour, chickpea protein-enriched fraction, and yeast were first blended in a bowl, followed by adding 30 °C water with salt dissolved. The mixture was kneaded for 9 min by hand until a consistent dough was formed. Proofing was performed in a 30 °C electric oven (Memmert GmbH, Germany) for two times and each proofing step took 1 h. After the first proofing, the dough was taken out once for shaping to expel extra air. The dough was baked at 200 °C. To following the baking process as function of time, multiple breads were baked for 30 min and samples were taken out with a 5-min time interval for evaluation. The correct baking time was established by evaluating the crust colour.

2.6. Evaluation of chickpea protein-enriched bread

2.6.1. Compositional analysis

The nitrogen content of the breads was determined using the Dumas method (FlashEA 1112 series, Thermo Scientific, The Netherlands). A nitrogen conversion factor of N × 5.71 was used for the calculation of the protein content. The oil, ash, and moisture contents of bread were determined by methods AACC 30–25.01 (1999), AACC 08–01 (1983), and AACC 44–15.02 (1999), respectively. The content of carbohydrate was calculated by the difference.

2.6.2. Raffinose family oligosaccharides (RFOs)

High-performance anion exchange chromatography (HPAEC) was used to analyze the content of raffinose, stachyose, and verbascose in

bread. A Dionex ICS-5000 system (Thermo Fisher Scientific Inc., USA) was used with a CarboPac PA1 (2 mm × 250 mm) guard column (Thermo Fisher Scientific Inc., USA) and a pulsed amperometric detector (HPAEC-PAD). The RFOs were extracted by dispersing 1 g of bread crumb in 30 ml of demi-water and stirring for 30 min. Subsequently, the extract was transferred to a tube and centrifuged at 10000 rpm for 5 min. A 500 μL of the supernatant was transferred into a 2 ml centrifuge tube and proteins were precipitated by adding 500 μl Carrez reagent (250 μl Carrez A followed by 250 μl Carrez B). After centrifugation at 10000 rpm for 5min, 200 μl of the supernatant was transferred into a vial (0.3 ml PP Short Thread Vial 32 × 11.6 mm clear, BGB, Switzerland) for analysis. Standard solutions in the range of 1–20 mg/L were prepared for sugar identification and quantification. Other parameters setpoints were: injection volume was 10 μL and the eluent flow rate was 0.3 ml/min. Chromeleon 7.0 was used for numerical integration of the peak surface. All HPAEC analyses were carried out in duplicate.

2.6.3. Colorimeter

The colour of the crumb and crust of the bread was analyzed with a CR-400 colorimeter (Konica Minolta Inc., Japan). Results were expressed as CIE L*, a*, and b* value, in which L* represents lightness component, a* represents green (−) and red (+) while b* value represents blue (−) and yellow (+) (Zhang et al., 2019). The colorimeter was corrected with a calibration plate before measuring. Each sample was measured at three different spots on crust and crumb, respectively.

2.6.4. Specific volume

The specific volume of bread was determined by AACC International Approved Methods 10–05.01 with some modifications (1999). Couscous was used in the displacement method. The bread was weighed 1 h after baking. The specific volume of the bread was calculated and expressed as mL/g.

2.6.5. Texture profile analysis

A texture analyzer (TA.XTplusC, Stable Micro Systems Ltd., UK) equipped with a cylindrical probe of 40 mm was used to analyze the textural properties of bread. A crumb cube (1 × 1 × 1 cm) cut from the bread center was compressed to up to 40% strain with a speed of 2 mm/s. Other parameters were set as: pre-speed 1 mm/s, post-speed 2 mm/s, trigger force 5 g, and a delay of 30 s between two compressions. The hardness, chewiness, and cohesiveness were determined. Measurements were conducted in duplicate for each bread.

2.6.6. C-cell analysis

The cellular structure of bread was analyzed with a C-Cell imaging system (Calibre Control International Ltd., Warrington, UK). The analysis of the bread samples was performed immediately after slicing with a rotary disc blade cutter (Sroan, Bean, & MacRitchie, 2009). Parameters determined include the surface area, cell diameter, and cell wall thickness.

2.6.7. Confocal laser scanning microscopy (CLSM)

Frozen dough samples were cut into 60 μm thick slices with a cryomicrotome (CR 50-H Bio-med, Heidelberg) and placed on a glass slide. An aqueous solution of rhodamine B (0.002%, w/v) and fluorescein isothiocyanate (FITC) (0.05%, w/v) was used for staining the gluten and starch, respectively. A cover slip was dropped carefully to avoid air bubbles. After staining in dark conditions for 30 min, an LSM 510-META confocal microscopy (Carl ZEISS, Germany) was used for observation. The λ_{exc} and λ_{emi} of FITC were 488 nm and 525 nm. The λ_{exc} and λ_{emi} of rhodamine B were 543 nm and 627 nm.

2.6.8. Microbiological properties

The microbiological stability of wheat bread and chickpea fortified bread was evaluated following Belz et al. (2019) with some modifications. Bread samples were sliced, and 5 pieces of each bread were exposed to air for 10 min. Each of the slices was then packaged in ziplocked polyethylene bags and stored at 30 °C for 5 days for the spoilage experiment, and slices were sampled every 24 h. One gram of sample was serially diluted in 9 ml PPS. Subsequently, 50 μl of dilution was spread out on plate count agar (PCA) (Merck, Germany) plates using a spiral plater (Eddy Jet IUL, Neutec Group Inc., USA). The plates were incubated at 30 °C for 24 h.

2.7. Statistics analysis

All analyses were carried out in duplicate unless indicated differently. Means and standard deviations were calculated using SPSS (Version 22.0, IBM, USA) statistical software. One-way ANOVA was performed to evaluate the effect of chickpea protein-enriched fraction and its sourdough addition on properties of bread. Duncan's test at a 95% confidence level was applied to verify the differences between groups.

3. Results and discussions

3.1. Recipe development

Partial replacement of the wheat flour by a chickpea protein-enriched fraction enhances the protein content in final bread products, but since chickpea protein has different properties compared to wheat gluten such as solubility and water holding capacity (Jagannadham, Parimalavalli, Babu, & Rao, 2014), the fortification will influence the dough properties. Specifically, the amount of water in the recipe of chickpea-fortified bread should be determined. Farinograph measurements were applied to establish the appropriate amount of water at substitution levels of 20% and 30%. The dough development of composite chickpea-wheat flour mixtures is affected due to the fortification with the chickpea fraction, which is high in protein and non-starch polysaccharides (e.g. pentosan) (Mohammed, Ahmed, & Senge, 2014). The wheat flour is diluted by the introduction of the fraction, while adding the right amount of water is crucial for the development of the gluten network (Mohammed et al., 2014). A farinograph was used to record the resistance to deformation of the dough during mixing. This was done to establish the optimal ratio of water to flour (leading to a dough resistance of 500 BU) and record the dough development time and dough stability. The water sorption decreased with more chickpea from 61.9% to 52.9% (Table 2), which means less water was needed in the dough to achieve the same dough consistency during mixing/kneading with chickpea fraction fortified. If the water addition was kept the same as for wheat dough, the doughs with chickpea fraction yielded a very sticky dough that was difficult to knead. The high water sorption of gluten compared to other proteins explains why less water is needed with high-protein chickpea dough (Yousseff, Salem, & Abdel-Rahman, 1976). Earlier, a decrease in water sorption was also observed for wheat flour that was partially substituted with lentil flour (Portman et al., 2018). The dough-development time gradually

Table 2
Farinograph data of wheat dough and doughs fortified with 20% or 30% chickpea protein-enriched fraction. Data marked with different lowercase superscript in the same column indicate significant differences ($P < 0.05$).

	Water absorption (ml/100 g)	Dough-development time (min)	Dough stability (min)
Wheat dough	$61.9^{b} \pm 0.3$	$1.5^{a} \pm 0.1$	$4.2^{a} \pm 0.1$
Dough fortified with 20% chickpea protein-enriched fraction	$57.2^{ab} \pm 1.2$	$3.0^{ab} \pm 0.5$	$14.0^{b} \pm 0.5$
Dough fortified with 30% chickpea protein-enriched fraction	$52.9^{a} \pm 2.9$	$3.6^{b} \pm 0.6$	$12.9^{b} \pm 0.7$

increased as wheat flour was replaced by the chickpea protein-enriched fraction, indicating the dough took more time to reach its maximum consistency. This is due to a weakening of the gluten network due to dilution and hydration. At the same time, the dough stability time increased after fortification with the chickpea fraction. According to Zafar, Allafi, Alkandari, and Al-Othman (2020), the glycoprotein (lectin, protease inhibitor) presented in chickpea protein-enriched (fine) fraction are responsible for the improved dough stability time. Based on the farinograph results, the adjusted recipes for bread with 20% and 30% chickpea protein-enriched fraction were determined (Table 1). The recipe for chickpea sourdough bread is similar to the normal one, except that less water is added to compensate for water added for the sourdough fermentation at a later stage.

3.2. Browning as function of baking time

Surface browning of bread is caused by non-enzymatic browning including Maillard reaction, which is an important indicator of the bread quality. To follow the browning of the breads as function of the baking time, bread was baked at 200 °C. As shown in Fig. 1, the colour of the crust became browner with longer baking time. Bread prepared with chickpea had a significantly darker colour than pure wheat bread (control) and the colour deepened as the level of substitution increased. This is consistent with previous findings, where researchers attributed the colour change to the larger lysine content of chickpea. Lysine is the most active essential amino acids to react with reducing sugar in the Maillard reaction (Mohammed et al., 2014). Moreover, fructose, glucose, and sucrose can caramelize as the temperature of the crust approaches the oven temperature, adding to the colouration (Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001; Zhang, Taal, Boom, Chen, & Schutyser, 2018). The formation of brown pigments gave the breads a yellow-brown colour. Interestingly, it was observed that with the same substitution level, the breads prepared with fermented chickpea protein-enriched fraction (sourdough) had a somewhat lighter colour than the unfermented ones, with the differences becoming larger as the baking time increased (Fig. 1). This can be explained by the metabolization of reducing sugars like glucose and fructose by the LAB during fermentation (Hatzikamari, Kyriakidis, Tzanetakis, Biliaderis, & Litopoulou-tzanetaki, 2007; Xing et al., 2020), and thus a lower availability of reactants for the Maillard reaction. Besides, a lower pH can slow the Maillard reaction and the citric acid that is produced during fermentation may de-colour phenolic compounds like catechins and tannins which are naturally present in chickpea, and that are responsible for the characteristic yellow colour (Ajandouz et al., 2001; Güémes-Vera, Peña-Bautista, Jiménez-Martínez, Dávila-Ortiz, & Calderón-Domínguez, 2008). In further experiments, a baking time of 20 min was selected. Baking for longer than 20 min caused too extensive browning of the crust, which decreases the acceptability of the chickpea-enriched bread (Castro, Oblitas, Chuquizuta, & Avila-George, 2017).

3.3. Nutritional properties

3.3.1. Protein content

A chickpea protein-enriched fraction produced by air classification had a protein content of 31.4 g/100 g on dry basis, which is 57% higher than that of chickpea flour. Therefore, wheat flour replacement by chickpea protein-enriched fraction is more efficient compared to chickpea flour in terms of protein improvement. In comparison to wheat bread, the protein content of bread prepared with 20% and 30% of chickpea protein-enriched fraction was increased by 26.5% and 38.5%, respectively (Fig. 2). The chickpea breads have improved protein content compared to wheat bread and may be classified as "source of protein" (requiring that at least 12% of the energy value of food is provided by protein) according to Regulation (EC) No 1924/2006. Bread fortified with chickpea sourdough showed similar protein content as found with the unfermented ones at the same substitution level. The composition of the breads are summarised in Table 3.

3.3.2. Content of oligosaccharides

The presence of flatulence-causing oligosaccharides reduces the acceptability of chickpea fortified bread (Veenstra et al., 2010). Solid-state fermentation can reduce the content of oligosaccharides in chickpea fraction (Galli, Venturi, Pini, Guerrini, & Granchi, 2019). Besides, oligosaccharides are not very heat-stable and start decomposing above 200 °C (Forgo, Kiss, Korózs, & Rapi, 2013), combining fermentation and heat treatment can contribute to a large reduction of the oligosaccharides (Khattab et al., 2009). Fig. 3 shows that with the use of chickpea sourdough instead of the same unfermented fraction, the content of raffinose, stachyose, and verbascose decreased by 75.4 ± 0.3%, 97.6 ± 0.8%, and 90.0 ± 2.1%, respectively. This means that a much larger intake will be needed to cause flatus. Earlier studies reported a reduction of 63.2% for oligosaccharides of the raffinose family in bread prepared with chickpea sourdough in which a foreign *Lactobacillus plantarum* strain was used (Galli et al., 2019). In our study the reduction was much higher because *Pediococcus acidilactici* were isolated

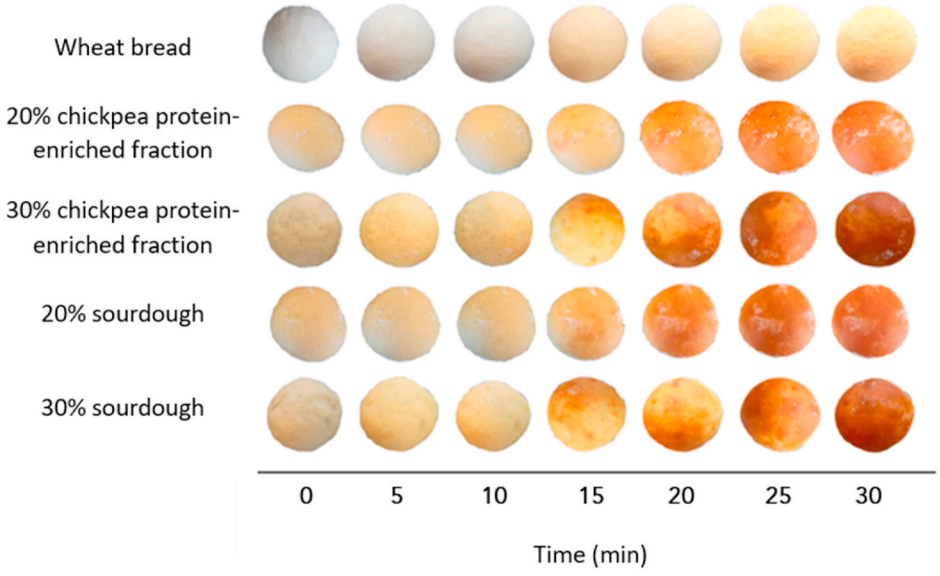


Fig. 1. The colour change of bread baked over time (up to 30 min) at 200 °C. Rows from the top to the bottom represent: wheat breads, breads fortified with 20% chickpea protein-enriched fraction, breads fortified with 30% chickpea protein-enriched fraction, breads fortified with 20% chickpea sourdough, and breads fortified with 30% chickpea sourdough, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

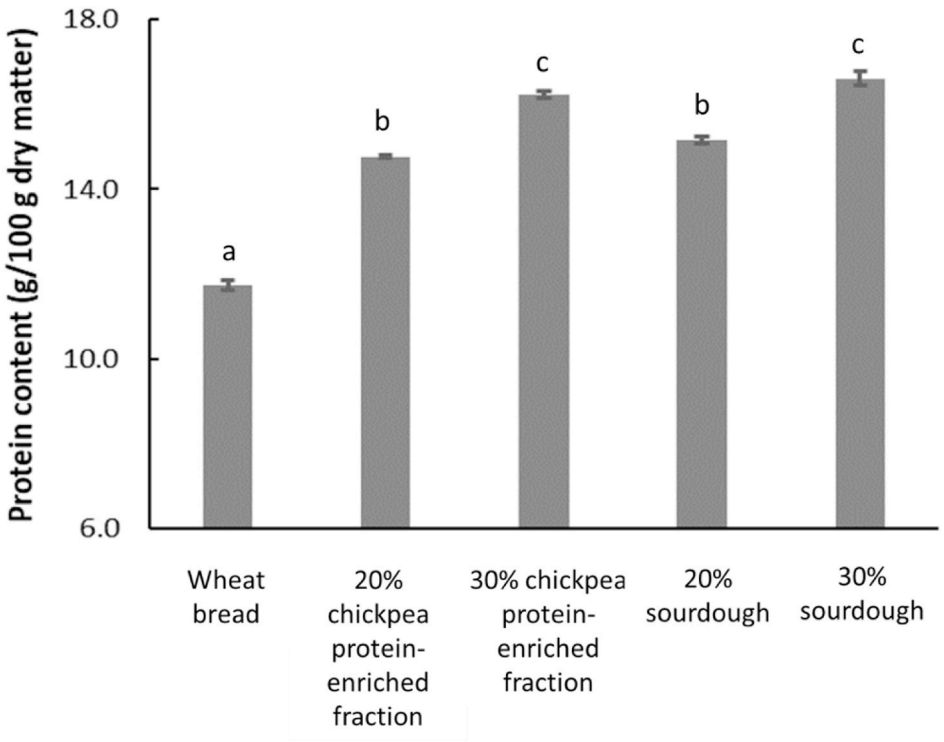


Fig. 2. The protein content of wheat bread (control) and bread fortified with chickpea protein-enrich fraction or its sourdough at substitution levels of 20% and 30%. Data marked with a different lowercase superscript in the same column indicate significant differences ($P < 0.05$).

Table 3
Summary of the composition of the breads on dry basis.

	Protein (g/100 g)	Carbohydrate (g/100 g)	Oil (g/100 g)	Ash (g/100 g)
Wheat bread	$11.7^{a} \pm 0.1$	$85.4^{a} \pm 2.6$	$0.8^{a} \pm 0.1$	$2.1^{a} \pm 0.0$
20% protein-enriched fraction	$14.8^{b} \pm 0.0$	$81.0^{a} \pm 3.6$	$1.7^{b} \pm 0.0$	$2.6^{b} \pm 0.0$
30% protein-enriched fraction	$16.2^{c} \pm 0.1$	$78.2^{a} \pm 3.0$	$2.6^{c} \pm 0.1$	$2.9^{c} \pm 0.1$
20% sourdough	$15.2^{b} \pm 0.1$	$80.8^{a} \pm 2.7$	$1.5^{b} \pm 0.1$	$2.6^{b} \pm 0.1$
30% sourdough	$16.6^{c} \pm 0.2$	$78.1^{a} \pm 2.3$	$2.4^{c} \pm 0.1$	$2.9^{c} \pm 0.1$

from chickpea itself (Xing et al., 2020), thus are best adapted to the substrate. The levels of oligosaccharides in chickpea sourdough bread in fact were close to that of wheat bread, indicating that the fermentation effectively removes these anti-nutritional factors in legume fortified bread to acceptable levels.

3.4. Physical properties

3.4.1. Colour

The colour of the bread crust and crumb was expressed in CIE L*, a*, and b* values, corresponding to brightness, green (−)/red (+), and blue (−)/yellow (+), respectively. The colour difference (ΔE) of the bread was calculated with the standard being the wheat bread. The L* value of the crust decreased and the a* and b* parameters increased, as wheat flour replaced by chickpea ingredients (Fig. 4A), indicating that a redder and yellower crust was obtained for chickpea fortified bread. The darker crust colour was attributed to increased Maillard reaction due to more reducing sugar and higher lysine contents (Mohammed, Ahmed, & Senge, 2012; Yousseff et al., 1976). However, the colour difference caused by different substitution levels was not obvious until after 20 min baking time. As it is also demonstrated in Fig. 1, the effect of the

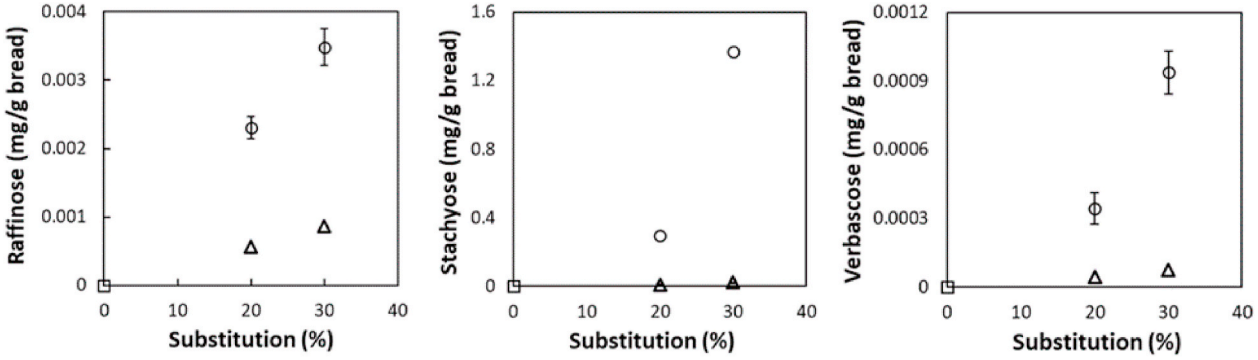


Fig. 3. The content of oligosaccharides (raffinose, stachyose, and verbascose) in wheat bread (□), bread fortified with chickpea protein-enriched fraction (○), and its sourdough (Δ) at substitution levels of 20% and 30%. The error bars represent the standard deviation. Some error bars are not visible due to the small standard deviation values.

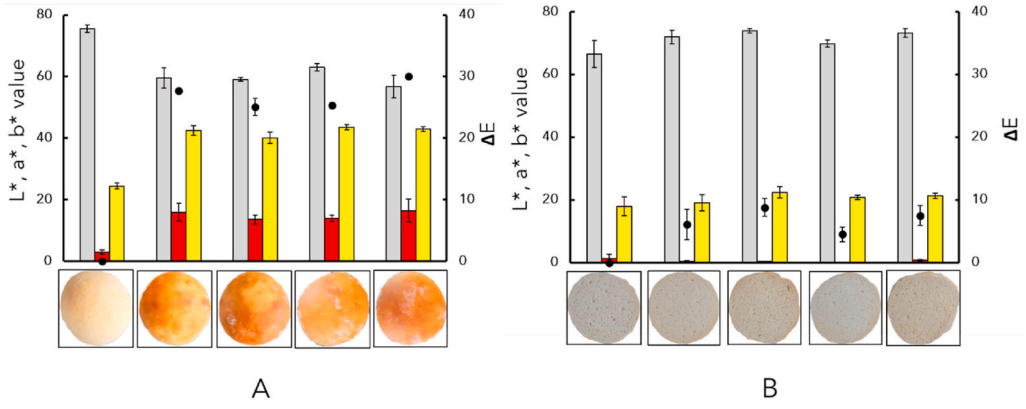


Fig. 4. CIE L* (□), a* (■), b* (□) of bread crust (A) and crumb (B), and the colour difference ΔE (●) between the wheat bread (control) and different substitutions. The error bar represents the standard deviation. Photos of corresponding crust and crumb are shown beneath the bar charts. From left to right: wheat bread (control), bread fortified with 20% chickpea protein-enriched fraction, 30% chickpea protein-enriched fraction, 20% chickpea sourdough, and 30% chickpea sourdough, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

fermentation on browning was more significant when baked at a longer time (30 min). The crumb was less red and yellow compared to the crust, while differences between wheat bread and chickpea enriched breads were small (Fig. 4B). In summary, the substitution of chickpea protein-enriched fraction had a larger impact on the crust colour than on the crumb, and fermentation did not significantly affect the colour compared to that of the unfermented ones.

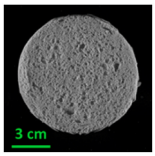
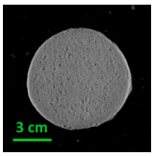
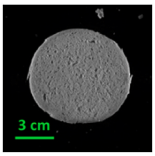
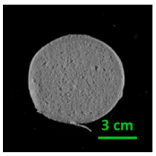
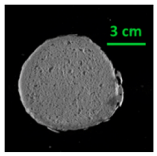
3.4.2. Other physical properties

An overview of the measured physical properties of the breads is provided in Table 4. The volume, cellular structure, and texture of bread changed upon fortification with the chickpea ingredients. In general, chickpea fortified bread had a significantly ($P < 0.05$) smaller specific volume than that of pure wheat bread. This can be explained by the weakened gluten structure and the decreased dough elasticity (Mohammed et al., 2012). The structure of the breads was visualized by the C-Cell imaging system, since the cellular structure contributes to bread appearance and texture (Millar, Barry-Ryan, Burke, McCarthy, & Gallagher, 2019). Pure wheat bread showed a large average cell diameter, suggesting a soft and fluffy crumb, while the chickpea fortified bread exhibited a smaller cell diameter and a higher total cell number per cm^2 indicating a denser crumb (Millar et al., 2019). The cell wall thickness of the cells in the crumb was found to decrease upon the fortification with chickpea, which coincided with a decrease in specific bread volume (Table 4). The weakened gluten network due to the introduction of the chickpea fractions led to thinner cell wall, lower gas retention and thus smaller breads (Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2015). Bread with chickpea fortification showed significantly ($P < 0.05$) higher hardness and the values increased as the substitution level increased (compared to wheat bread). The substitution levels did not show significant influence on chewiness and cohesiveness, except a significant increase in chewiness when sourdough was added. The bread fortified with sourdough had similar hardness and chewiness compared to the bread with unfermented chickpea protein-enriched fraction. As for cohesiveness, no effect of substitution levels or fermentation was observed.

3.4.3. Micro-structure of doughs and breads

CLSM was used to visualize the distribution of protein and starch in the dough matrix, which helps also to understand the effect of chickpea ingredients on the microstructure of the final bread. Starch was stained green by FITC and protein was stained red by rhodamine B. Since FITC is also reactive towards amino groups on proteins (Mariotti, Lucisano, Pagani, & Ng, 2009), proteins stain orange instead of red when the two dyes are used. In Fig. 5, the strand-like gluten structure was not observed directly (McCann & Day, 2013), yet the elongated protein clusters in the pure wheat dough (Fig. 5 a) are representative of it, and are more prominent than in the other doughs. The microstructure of the doughs prepared with different levels of chickpea ingredients is similar, with protein aggregates distributed evenly and a large number of small air bubbles. Replacing the chickpea protein-enriched fraction with its sourdough did not lead to any clear changes in the microstructure of the

Table 4
Cellular structure and texture of bread fortified with 20% and 30% chickpea protein-enriched fraction or its sourdough compared to the wheat bread (control). Data in the same row marked with different lower-case letters indicate significant differences ($P < 0.05$).

	Wheat bread (control)	20% chickpea protein-enriched fraction	30% chickpea protein-enriched fraction	20% chickpea sourdough	30% chickpea sourdough
Cross-section image					
Surface area (cm^2)	$66.88^{c} \pm 0.83$	$49.53^{b} \pm 0.29$	$44.03^{ab} \pm 1.65$	$42.30^{a} \pm 1.40$	$42.68^{a} \pm 2.98$
Cell diameter (mm)	$1.56^{b} \pm 0.00$	$0.96^{a} \pm 0.03$	$1.08^{a} \pm 0.06$	$1.07^{a} \pm 0.02$	$1.07^{a} \pm 0.09$
Cell wall thickness (mm)	$0.43^{a} \pm 0.01$	$0.37^{b} \pm 0.00$	$0.38^{b} \pm 0.01$	$0.37^{b} \pm 0.00$	$0.39^{b} \pm 0.00$
Total cell per cm^2	$79.9^{a} \pm 0.1$	$122.6^{c} \pm 3.6$	$110.8^{bc} \pm 4.8$	$121.3^{c} \pm 4.3$	$108.2^{b} \pm 2.2$
Specific volume (ml/g)	$2.44^{b} \pm 0.21$	$2.10^{ab} \pm 0.05$	$1.84^{a} \pm 0.03$	$1.95^{a} \pm 0.11$	$1.78^{a} \pm 0.04$
Hardness (N)	$6.01^{a} \pm 0.02$	$10.04^{b} \pm 1.50$	$13.86^{c} \pm 0.15$	$11.95^{bc} \pm 0.29$	$16.92^{d} \pm 1.63$
Chewiness (N)	$4.21^{a} \pm 0.01$	$6.06^{ab} \pm 0.58$	$7.62^{ab} \pm 0.37$	$10.50^{b} \pm 2.52$	$10.68^{b} \pm 1.55$
Cohesiveness (–)	$0.73^{b} \pm 0.00$	$0.68^{ab} \pm 0.03$	$0.64^{a} \pm 0.03$	$0.67^{ab} \pm 0.02$	$0.66^{ab} \pm 0.01$

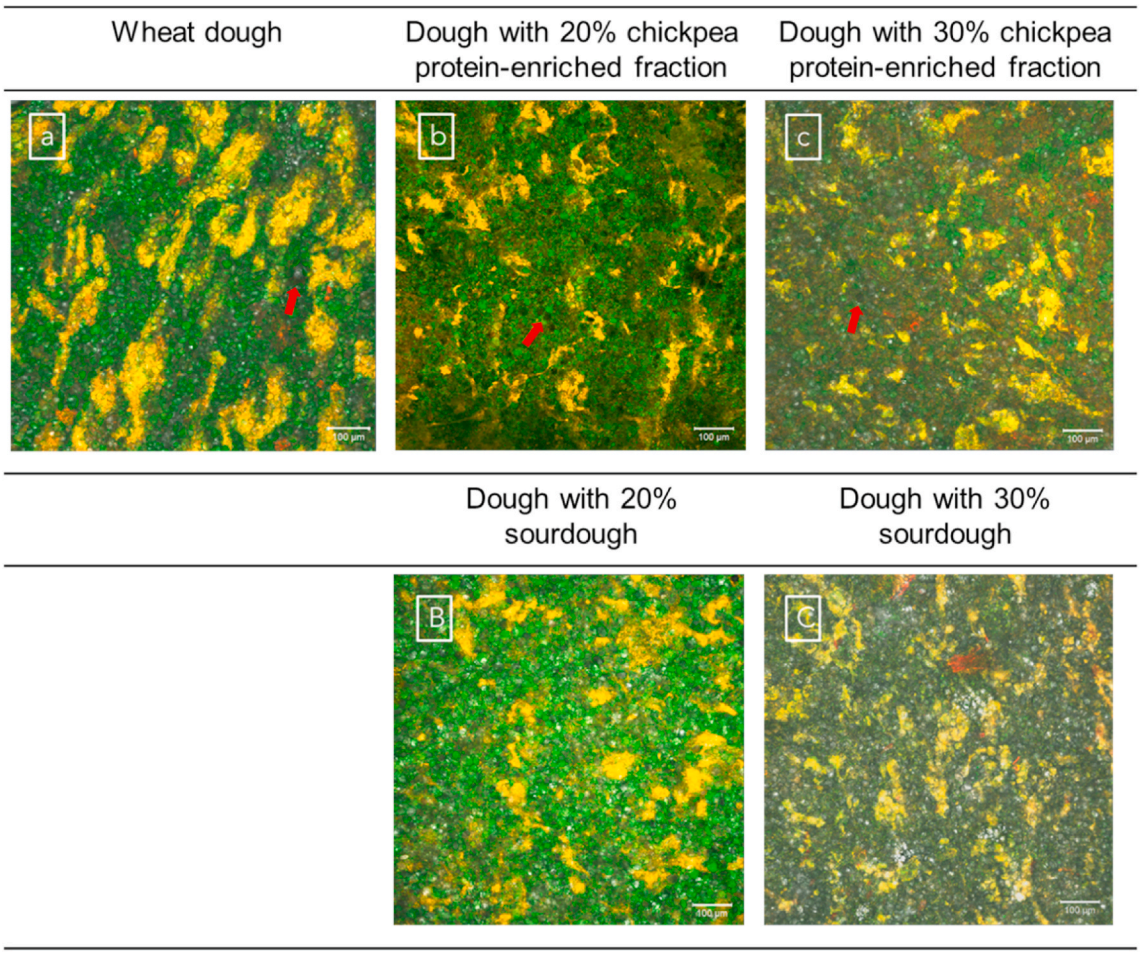


Fig. 5. CLSM pictures of wheat dough (a), wheat dough fortified with 20% (b) and 30% (c) chickpea protein-enriched fraction, and wheat dough fortified with 20% (B) and 30% (C) chickpea sourdough. Proteins are stained red/orange, starch granules are stained green, and air bubbles are in black. The white bar represents 100 μm. Red arrows indicate air bubbles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

doughs. Air is included during dough kneading and yielded a microstructure similar to that in a previous study (Bousquieres, Deligny, Riaublanc, & Lucas, 2014). All breads prepared with chickpea had a significantly smaller crumb gas cell diameter and a higher cell number density (Table 4). Because the doughs were pictured by CLSM before proofing, we expect that the difference in structure between wheat and chickpea enriched bread emerges during proofing.

3.5. Microbiological properties

In Fig. 6, the microbiological properties of bread with chickpea sourdough were compared with that of unfermented chickpea protein-enriched bread and wheat bread (control). Plate count agar was used in this study for the enumeration of spoilage bacteria of bread during a period of 5 days. The chickpea sourdough bread displayed the lowest colony number among all five samples during 5 days of storage (Fig. 6). Also, no mold growth was observed (results not shown). This is in contrast to the other breads where the total number of colonies dramatically increased to 5.3–5.7 log CFU/g bread within 24 h and reached 7.0–8.4 log CFU/g bread over five days.

The lower total colony count for chickpea sourdough bread is due to the accumulation of organic acids during LAB fermentation hampering the growth of spoilage microorganisms. The pH value of the dough partially replaced with chickpea sourdough was 4.5–4.6, while the pH of the other doughs was 5.4–5.5. Moreover, LAB strains produce antimicrobial substances like hydrogen peroxide and bacteriocin, inhibiting both Gram-positive and Gram-negative pathogens (Olatunde et al., 2018bib_Olatunde_et_al_2018). The substitution amount of the sourdoughs did not influence the total colony count within five days of storage, even though at higher sourdough replacements one may expect a stronger inhibition on microbial growth (Hendek Ertop & Coşkun,

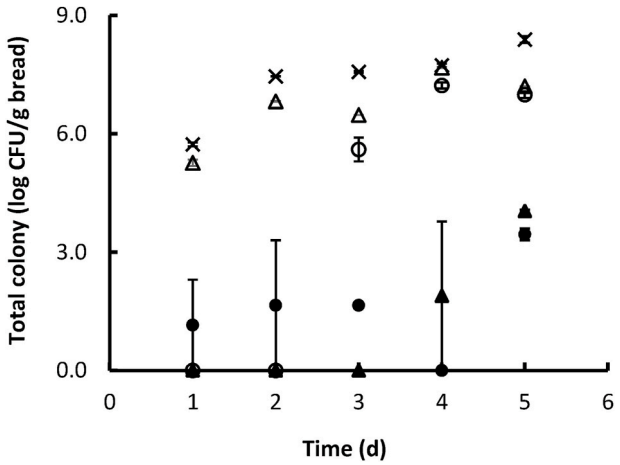


Fig. 6. Total colony count of wheat bread (control) (×), bread fortified with 20% chickpea protein-enriched fraction (△), 30% chickpea protein-enriched fraction (○), 20% sourdough (▲), and 30% sourdough (●) for a period of 5 days when stored at 30 °C. The error bars represent standard deviation.

2018).

4. Conclusions

Wheat bread could be fortified by using dry fractionated and fermented chickpea protein-enriched (fine) fraction. The level of flatulence-causing anti-nutritional factors was strongly reduced by solid-state fermentation. The addition of both chickpea protein-enriched fraction or its sourdough decreases the specific volume and increases the crumb hardness and firmness. Depending on the baking time, the sourdough yields less crust browning during baking, and less microbial spoilage over 5 days of storage. Based on these results, the dry fractionated, protein-enriched and fermented chickpea ingredient have potential as ingredient for developing protein-enriched bakery products, while keeping the low environmental footprint of the ingredients obtained with dry separation. It is worth noting that the bread formula proposed in the current study cannot guarantee that the product is appealing to consumers. For future related research, in order to compensate the deficiencies of texture and sensory quality, the formulation needs to be improved by adding natural quality improvers; more application ways of fermented protein-enriched fraction should be explored; and the starch-rich fraction should also be used in food development.

CRediT authorship contribution statement

Qinhui Xing: Conceptualization, Data curation, Formal analysis, Investigation, Writing - original draft. **Konstantina Kyriakopoulou:** Supervision, Validation, Writing - review & editing. **Lu Zhang:** Methodology. **Remko M. Boom:** Supervision, Writing - review & editing. **Maarten A.I. Schutyser:** Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

The authors have no declarations of interest.

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References

Aiking, H. (2014). Protein production: Planet, profit, plus people? *American Journal of Clinical Nutrition, 100*(suppl_1), 483S–489S.

Ajandouz, E., Tchiakpe, L., Ore, F. D., Benajiba, A., & Puigserver, A. (2001). Effects of pH on caramelization and Maillard reaction kinetics in fructose-lysine model systems. *Journal of Food Science, 66*(7), 926–931.

Asif, M., Rooney, L. W., Ali, R., & Riaz, M. N. (2013). Application and opportunities of pulses in food system: A review. *Critical Reviews in Food Science and Nutrition, 53*(11), 1168–1179.

Belz, M. C., Axel, C., Arendt, E. K., Lynch, K. M., Brosnan, B., Sheehan, E. M., et al. (2019). Improvement of taste and shelf life of yeasted low-salt bread containing functional sourdoughs using Lactobacillus amylovorus DSM 19280 and Weisella cibaria MG1. *International Journal of Food Microbiology, 302*, 69–79.

Boukid, F., Zannini, E., Carini, E., & Vittadini, E. (2019). Pulses for bread fortification: A necessity or a choice? *Trends in Food Science & Technology, 88*, 416–428.

Bousquieres, J., Deligny, C., Riaublanc, A., & Lucas, T. (2014). CLSM study of layers in laminated dough: Roll out of layers and elastic recoil. *Journal of Cereal Science, 60*(1), 82–91.

Castro, W., Oblitas, J., Chuquizuta, T., & Avila-George, H. (2017). Application of image analysis to optimization of the bread-making process based on the acceptability of the crust color. *Journal of Cereal Science, 74*, 194–199.

Forgo, P., Kiss, A., Korózs, M., & Rapi, S. (2013). Thermal degradation and consequent fragmentation of widely applied oligosaccharides. *Microchemical Journal, 107*, 37–46.

Foschia, M., Horstmann, S. W., Arendt, E. K., & Zannini, E. (2017). Legumes as functional ingredients in gluten-free bakery and pasta products. *Annual review of food science and technology, 8*, 75–96.

Galli, V., Venturi, M., Pini, N., Guerrini, S., & Granchi, L. (2019). Exploitation of sourdough lactic acid bacteria to reduce raffinose family oligosaccharides (RFOs) content in breads enriched with chickpea flour. *European Food Research and Technology, 245*(11), 2353–2363.

Güémes-Vera, N., Peña-Bautista, R. J., Jiménez-Martínez, C., Dávila-Ortiz, G., & Calderón-Domínguez, G. (2008). Effective detoxification and decoloration of Lupinus mutabilis seed derivatives, and effect of these derivatives on bread quality and acceptance. *Journal of the Science of Food and Agriculture, 88*(7), 1135–1143.

Hatzikamari, M., Kyriakidis, D. A., Tzanetakis, N., Biliaderis, C. G., & Litopoulou-tzanetaki, E. (2007). Biochemical changes during a submerged chickpea fermentation used as a leavening agent for bread production. *European Food Research and Technology Zeitschrift für Lebensmittel Untersuchung und Forschung. A, 224*(6), 715–723.

Hendek Ertop, M., & Coşkun, Y. (2018). Shelf-life, physicochemical, and nutritional properties of wheat bread with optimized amount of dried chickpea sourdough and yeast by response surface methodology. *Journal of Food Processing and Preservation, 42*(7), Article e13650.

Jagannadham, K., Parimalavalli, R., Babu, A. S., & Rao, J. S. (2014). A study on comparison between cereal (wheat) and non cereal (chickpea) flour characteristics. *International Journal Current Trend Research, 3*(2), 70–76.

Khattab, R., & Arntfield, S. (2009). Nutritional quality of legume seeds as affected by some physical treatments 2. Antinutritional factors. *LWT-Food Science and Technology, 42*(6), 1113–1118.

Mariotti, M., Lucisano, M., Pagani, M. A., & Ng, P. K. (2009). The role of corn starch, amaranth flour, pea isolate, and Psyllium flour on the rheological properties and the ultrastructure of gluten-free doughs. *Food Research International, 42*(8), 963–975.

Mattila, P., Mäkinen, S., Eurola, M., Jalava, T., Pihlava, J.-M., Hellström, J., et al. (2018). Nutritional value of commercial protein-rich plant products. *Plant Foods for Human Nutrition, 73*(2), 108–115.

McCann, T. H., & Day, L. (2013). Effect of sodium chloride on gluten network formation, dough microstructure and rheology in relation to breadmaking. *Journal of Cereal Science, 57*(3), 444–452.

Millar, K., Barry-Ryan, C., Burke, R., McCarthy, S., & Gallagher, E. (2019). Dough properties and baking characteristics of white bread, as affected by addition of raw, germinated and toasted pea flour. *Innovative Food Science & Emerging Technologies, 56*, 102189.

Mohammed, I., Ahmed, A. R., & Senge, B. (2012). Dough rheology and bread quality of wheat–chickpea flour blends. *Industrial Crops and Products, 36*(1), 196–202.

Mohammed, I., Ahmed, A. R., & Senge, B. (2014). Effects of chickpea flour on wheat pasting properties and bread making quality. *Journal of Food Science & Technology, 51*(9), 1902–1910.

Nkhabutlane, P., du Rand, G. E., & de Kock, H. L. (2014). Quality characterization of wheat, maize and sorghum steamed breads from Lesotho. *Journal of the Science of Food and Agriculture, 94*(10), 2104–2117.

Olatunde, O. O., Obadina, A. O., Omemu, A. M., Oyewole, O. B., Olugbile, A., & Olukomaiya, O. O. (2018). Screening and molecular identification of potential probiotic lactic acid bacteria in effluents generated during ogi production. *Annals of Microbiology, 68*(7), 433–443.

de Oliveira, L. M., da Silva Lucas, A. J., Cadaval, C. L., & Mellado, M. S. (2017). Bread enriched with flour from cinereous cockroach (Nauphoeta cinerea). *Innovative Food Science & Emerging Technologies, 44*, 30–35.

Portman, D., Blanchard, C., Maharjan, P., McDonald, L. S., Mawson, J., Naiker, M., et al. (2018). Blending studies using wheat and lentil cotyledon flour—effects on rheology and bread quality. *Cereal Chemistry, 95*(6), 849–860.

Schutyser, M., Pelgrom, P., Van der Goot, A., & Boom, R. (2015). Dry fractionation for sustainable production of functional legume protein concentrates. *Trends in Food Science & Technology, 45*(2), 327–335.

Shrivastava, C., & Chakraborty, S. (2018). Bread from wheat flour partially replaced by fermented chickpea flour: Optimizing the formulation and fuzzy analysis of sensory data. *Lebensmittel-Wissenschaft und -Technologie, 90*, 215–223.

Sokolowski, C. M., Higgins, S., Vishwanathan, M., & Evans, E. M. (2019). The relationship between animal and plant protein intake and overall diet quality in young adults. *Clinical Nutrition, 39*(8), 2609–2616.

Sroan, B. S., Bean, S. R., & MacRitchie, F. (2009). Mechanism of gas cell stabilization in bread making. I. The primary gluten–starch matrix. *Journal of Cereal Science, 49*(1), 32–40.

Veenstra, J., Duncan, A., Cryne, C., Deschambault, B., Boye, J., Benali, M., et al. (2010). Effect of pulse consumption on perceived flatulence and gastrointestinal function in healthy males. *Food Research International, 43*(2), 553–559.

Villarino, C. B. J., Jayasena, V., Coorey, R., Chakrabarti-Bell, S., & Johnson, S. K. (2015). The effects of Australian sweet lupin (ASL) variety on physical properties of flours and breads. *LWT-Food Science and Technology, 60*(1), 435–443.

Xing, Q., Dekker, S., Kyriakopoulou, K., Boom, R. M., Smid, E. J., & Schutyser, M. A. (2020). Enhanced nutritional value of chickpea protein concentrate by dry separation and solid state fermentation. *Innovative Food Science & Emerging Technologies, 59*, 102269.

Yousseff, S. A., Salem, A., & Abdel-Rahman, A. H. Y. (1976). Supplementation of bread with soybean and chickpea flours. *International Journal of Food Science and Technology, 11*(6), 599–605.

Zafar, T. A., Allafi, A. R., Alkandari, D., & Al-Othman, A. (2020). Rheological characteristics of wheat–chickpea composite flour doughs and effect of Amla powder

(Phyllanthus emblica L.) addition on the functional properties of bread. *Food Science and Technology International*, 1082013220950068.

Zhang, L., Taal, M. A., Boom, R. M., Chen, X. D., & Schutyser, M. A. (2018). Effect of baking conditions and storage on the viability of Lactobacillus plantarum supplemented to bread. *Lebensmittel-Wissenschaft und -Technologie, 87*, 318–325.

Zhang, L., van Boven, A., Mulder, J., Grandia, J., Chen, X. D., Boom, R. M., et al. (2019). Arabinoxylans-enriched fractions: From dry fractionation of wheat bran to the investigation on bread baking performance. *Journal of Cereal Science, 87*, 1–8.