**Functional and nutritional replacement of gluten in gluten-free yeast-leavened breads
by using β -conglycinin concentrate extracted from soybean flour**

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Abstract

The search of proteins that could act as both structural agents and nutritional enhancers in gluten free bread is still a challenge for the food industry. The present study evaluated the inclusion of 10% β -conglycinin concentrate (β CC) extracted from defatted soybean flour to rice flour to improve the structure and protein quality of gluten-free yeast-leavened bread. β CC breadmaking performance was compared to vital gluten functionality. Batter pasting properties and bread characterization in terms of color, texture, image analysis, microstructure and protein quality were assessed. β CC and gluten led to batters with higher peak viscosity and breakdown, although only β CC was able to increase the setback. The inclusion of 10% β CC in rice flour formulations led to breads with improved color parameters and protein quality. The texture properties of β CC breads did not present significant differences compared to vital gluten breads. The crumb analysis showed that β CC led to lower cell density with highest mean cell area. The micrographs showed that β CC was able to create a net-like structure similar to the one created by gluten, confirming that β CC is capable of acting as structuring agent and protein quality improver in gluten-free formulations.

Keywords: β -conglycinin, proteins, gluten-free, bread, rice flour, protein quality.

1. Introduction

The baking properties of wheat flour are determined by the unique characteristics of its proteins, namely gluten. After hydration and kneading of wheat flour, gluten proteins form a network responsible of the cohesiveness, viscosity and elasticity of the dough, making it capable of holding the gas produced during fermentation and leading to the characteristic foam structure of bread (Veraverbeke & Delcour, 2002). However, these proteins are not considered safe for patients with celiac disease, which is the most common food intolerance of the world population (Wieser & Koehler, 2008). In these patients, gluten ingestion triggers an immune mediated response that causes villous atrophy in the small intestine and subsequent malabsorption. Therefore, celiac disease is frequently associated with deficiencies of macro and micronutrients (Theethira, Dennis, & Leffler, 2014). The most efficient treatment for celiac is a strict withdrawal of gluten containing foods from the diet (Wieser & Koehler, 2008). However, gluten-free baked goods are generally produced with refined starch, leading to products with low amounts of proteins, dietary fiber and micronutrients in comparison with their gluten product counterparts (Matos Segura & Rosell, 2011; Taylor & Rosell, 2016). Hence, the nutritional deficiencies of the celiac patients cannot be mitigated with the current gluten-free products unless those are nutritionally closer to their gluten containing equivalents.

Rice flour is one of the most common ingredients in the production of gluten-free foods including bread (Deora, Deswal, & Mishra, 2014) because it has bland taste, is colorless, inexpensive, easily digested, and present hypoallergenic properties (Capriles & Areas, 2014). However, due to the differences in its storage proteins, rice flour proteins are unable to develop a network with properties similar to gluten (Marco & Rosell, 2008a). To

improve the quality of the final products, several additives including hydrocolloids, enzymes, emulsifiers and proteins are supplemented in gluten-free formulations to increase the acceptability of consumers (Houben, Höchstötter, & Becker, 2012). In this backdrop, proteins have been evaluated in rice-flour formulations to overcome the problems associated to the lack of structure, mimic the characteristics that gluten provides to bread and also to enhance their nutritional value (Aprodu, Alexandra Badiu, & Banu, 2016; Marco & Rosell, 2008a; Patrascu, Banu, Vasilean, & Aprodu, 2017; Shin, Gang, & Song, 2010; Storck et al., 2013). Soybean has been one of the most evaluated sources of proteins for breadmaking due to their high lysine content, improving the essential amino acids content when combined with cereals (Tsen & Hoover, 1973), and more recently due to its potential to serve as structure-forming agent in gluten-free formulations (Marco & Rosell, 2008a, 2008b; Matos, Sanz, & Rosell, 2014; Miñarro, Albanell, Aguilar, Guamis, & Capellas, 2012; Sciarini, Ribotta, León, & Pérez, 2010; Ziobro, Witczak, Juszczak, & Korus, 2013). Nevertheless, in these studies the inclusion of additives such as hydrocolloids and enzymes was necessary to obtain the desired gluten-like network structure, since hitherto, no other protein has been able to fully replace gluten functionality.

In a recent model study, a β -conglycinin concentrate (β CC) proved to be an adequate structuring agent for yeast leavened breads produced with native corn starch (Espinosa-Ramírez, Garzon, Serna-Saldivar, & Rosell, 2018). The β CC was evaluated in a lean system where no other additives were used, which resulted in breads with higher 2D area, height, softness and cohesiveness compared to vital gluten breads. These results showed the potential of the β CC to improve the rise and quality properties of other gluten-free formulations acting as structure-forming agent. Moreover, since this β CC was obtained

after the fractionation of soybean proteins (Qi, Venkateshan, Mo, Zhang, & Sun, 2011), it could also contribute to improve the nutritional quality of rice flour breads.

The objective of this study was to evaluate the potential of β CC to mimic the gluten functionality in rice flour gluten-free leavened breads and to enhance the nutritive value of breads in terms of protein content and quality. Other structuring agents such as hydrocolloids or enzymes were not used during the process. Doughs and breads produced with rice flour and β CC or vital gluten were compared.

2. Material and methods

2.1 Materials

The rice flour was purchased from Harinera La Meta S.A (Lleida, Spain) (7.4% protein; 12.5% moisture). Vital gluten (77.4% protein; 8.3% moisture) was donated by Puratos (Groot-Bijgaarden, Belgium), whereas the β -conglycinin concentrate (β CC) was obtained according to the method proposed by Qi et al. (2011) with modifications reported by (Espinosa-Ramírez et al., 2018). The protein content of the lyophilized β CC containing 6.3% moisture was 87.5% (N*6.25).

Three formulations were tested for bread production: 1) 100% rice flour (negative control), 2) 90% rice flour + 10% vital gluten (positive control), and 3) 90% rice flour + 10% β CC (experimental treatment). Vital gluten and β CC were blended with rice flour in a proportion 90:10 based on the results obtained in a previous study, where the substitution of corn starch with 10% β CC improved the height and texture of gluten-free breads (Espinosa-

Ramírez et al., 2018). Gluten was substituted in the same concentration for comparison purposes.

2.2 Flours characteristics

The water binding capacity (WBC) of the rice flour and two composite flours was determined according to Method 44-15.02 (AACC, 1999). This value was selected as the optimal hydration level for the breadmaking process. Results were expressed as percentage in flour basis (g water/100 g flour) in accordance to baker's formulations. Five replicates were made for each sample.

The pasting and apparent viscosity properties of the batters were determined by Rapid Visco Analyzer (RVA 4500, Perten Instruments SA, Stockholm, Sweden) following the method reported by Xing, Niu, Su, & Yang (2016). Batters were prepared according to baker's formulation with 100% flour, 1.5% salt, 1.0% sugar and the hydration selected from the WBC value for each flour. For the analysis, batters were diluted (8 g of bread batter and 12 g of water) and held for 60 s at 50 °C, then heated from 50 to 95 °C at 12 °C min⁻¹ and held at 95 °C for 60 s. Thereafter, the batters were cooled to 50 °C at 12 °C min⁻¹ and held at 50 °C for 2 min. The mixing speed was 600 rpm and the apparent viscosity was recorded during the heating-cooling cycle using Thermocline software for Windows (Perten Instruments SA). The onset temperature, peak viscosity at 95 °C, breakdown (difference between peak viscosity and trough viscosity), final viscosity and setback (difference between final viscosity and trough viscosity) were evaluated. The RVA determination was conducted twice for each batter.

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122 2.3 Breadmaking process

123 For breadmaking, the lean baker's formulation consisted of 100% flour, 1.5% salt, 1.0%
124 sugar and 1.0% dry yeast. The water hydration used was selected from the WBC value
125 determined for each flour. Breadmaking was carried out following the mini-scale procedure
126 described by Garzon, Rosell, Malvar, & Revilla (2017). Briefly, doughs were kneaded at
127 100 rpm for 90 s in a stirrer with a turbine accessory (IKA Eurostar 40, Staufen, Germany).
128 After kneading, 2 g of dough were set in greased glass pans (diameter, 1.8 cm; height, 3
129 cm) and proofed for 40 min at 35 °C and 65% relative humidity. After fermentation, 100
130 µL of distilled water were added to the surface of the proofed dough to avoid dough surface
131 dryness and then baked in an oven set at 130 °C for 10 min. Two batches were run for each
132 formulation.

133

134 2.4 Bread characterization

135 Resulting breads were characterized in terms of color, texture and internal bread crumb
136 structure. The color of the crumb was determined using a colorimeter (Chroma Meter CR-
137 400/410, Konica Minolta, Tokyo, Japan) after white calibration ($L^* = 96.9$, $a^* = -0.04$, $b^* =$
138 1.84). Results were means of 4 replicates and expressed using the CIE- $L^*a^*b^*$ scale, where
139 L^* indicates lightness, a^* redness to greenness, and b^* yellowness to blueness.

140 The texture parameters were determined according to Garzon et al. (2017) in a Texture
141 Analyzer TA-XT2i (Stable Micro Systems, Surrey, UK) using a texture profile analysis
142 (TPA) double compression test. Bread slices of 10 mm thickness were compressed to 50%

of their original height at a test speed of 1 mm s⁻¹ with a stainless-steel probe of 25 mm diameter. Texture was assayed in four slices of each batch. The parameters evaluated were hardness, springiness, cohesiveness, chewiness, and resilience.

The 2D area of breads and their crumb cell structure were evaluated by digital image analysis. For that, eight breads of each formulation were selected and cut with a scalpel to obtain longitudinal and cross section images captured at 1200 ppi using a flatbed scanner HP Scanjet G3110 (Hewlett-Packard, USA). A 10x10 mm field of view was evaluated for each image. Image were improved by Image J software (National Institutes of Health, Bethesda, MD, USA) using the Otsu's algorithm for assessing the threshold (Gonzales-Barron & Butler, 2006). Data derived from the crumb structure analysis included: cell density (u/cm²), mean cell area (mm²) and cell circularity.

2.5 Protein quality of breads

The protein content of breads was determined according to the official method AOAC 920.87 (AOAC, 1990). The *in vitro* protein digestibility (IVPD) of lyophilized breads was assayed by the methods of Hsu, Vavak, Satterlee, & Miller (1977) and Bilgiçli, Ibanoglu, & Herken, (2007) with some modifications. Briefly, 1 mL of aqueous protein suspension containing 6.25 mg protein/mL was prepared. Samples were placed in a 37 °C water bath and the pH was adjusted to 8.00 using 0.1 N NaOH and/or 0.1 N HCl, while stirring. Trypsin at a concentration of 1.6 mg/ml was maintained in an ice bath and the pH was adjusted to 8.00 with 0.1 N NaOH and/or 0.1 N HCl. Then, 0.1 mL enzyme solution was added to the protein suspension, which was continuously stirred at 37 °C. The trypsin had

an activity of 13,766 BAEE units/mg proteins. The pH drop was recorded at 15 s after the addition of the enzymatic solution and at 1-min intervals during 10 min. The IVPD percentage was calculated using Eq. (1) (Hsu et al., 1977), where x is the change in pH after 10 min.

$$y = 210.464 - 18.1 x \quad \text{Eq. (1)}$$

Amino acids analysis of the flours used for breadmaking was performed using the official method AOAC 982.30 (AOAC, 1990) coupled to an HPLC system. Protein digestibility corrected amino acid scores (PDCAAS) were calculated according to WHO/FAO/ONU (2007) using the formulas:

$$\text{Limiting amino acid score} = \frac{\text{Limiting essential amino acid content of test protein}}{\text{Amino acid requirement pattern}} \quad \text{Eq. (2)}$$

$$\text{PDCAAS} = (\% \text{ protein digestibility} * \text{amino acid score}) / 100 \quad \text{Eq. (3)}$$

2.6 Scanning electron microscopy

For scanning electron microscopy (SEM), fermented dough and bread samples were frozen in liquid nitrogen immediately after production and freeze-dried. Doughs and breads were fractured and placed on a metal stub. Then samples were sputter-coated with gold and palladium by ion sputter (Bio-Rad SC-500, Aname, Madrid, Spain). Sample images were recorded with a Hitachi S-4800 (Ibaraki, Japan) scanning electron microscope and observed at 10kV accelerating voltage with magnification level of 700x.

2.7 Statistical analysis

Analysis of variance (ANOVA) was used for the statistical analysis of the results. Means were compared using Tukey's test. Analyses were performed using Minitab 17 statistical software and 95% confidence.

3. Results and Discussion

3.1 Flours and batters' characteristics

Due to the important role of water in the quality of gluten-free bread products (Aprodu et al., 2016), the water binding capacity (WBC) of rice flour and blends was determined to select the optimum water absorption for the breadmaking process. The formulation containing 10% β CC presented the lowest WBC (Table 1). According to a previous work, the WBC of β CC was significantly lower compared to vital gluten (0.98 g/g vs 1.45 g/g) (Espinosa-Ramírez et al., 2018). Therefore, the lower WBC of the blends containing β CC was attributed to the lower quantity of water bound by the β CC in comparison to rice flour and vital gluten. Crockett, Ie, & Vodovotz (2011) added higher amounts of water in breadmaking formulations of rice flour supplemented with a soybean protein isolate. The WBC of a soybean protein isolate could be up to 5 times higher (Paredes-Lopez, Ordorica-Falomir, & Olivares-Vazquez, 1991) when compared to the β CC used herein. Differences in the WBC according to the ingredients used in the formulation, highlight the importance of its assessment for each breadmaking system.

The RVA parameters and curves were obtained to evaluate the effect of the replacement of rice flour with proteins on the pasting properties of batters (Table 1; Fig. 1). The onset pasting temperature did not show significant differences among flours ranging from 66.4

°C to 68.1 °C. The onset pasting temperature was governed by the starch type and coincided with the pasting temperature of rice flour (Garzon et al., 2017; Heo, Jeon, & Lee, 2014). Peak viscosity was higher for batters containing 10% proteins. When the final viscosity was evaluated, the batter composed of rice flour and gluten showed the lowest value. Other authors found that the rice flour replacement with protein isolates (5%) resulted in lower peak and final viscosity (Marco & Rosell, 2008b; Storck et al., 2013). These authors related the lower final viscosity of protein-containing batters with the lower amount of gelatinized starch resulting from the partial replacement of the rice flour. Nevertheless, present results indicated that batters' performance is different than when only rice flour was tested. Conversely, batters containing 10% β CC presented higher values for final viscosity compared to those for gluten. Marco and Rosell (2008b) observed higher final viscosities when rice flour was supplemented with whey and egg proteins when compared to batters produced with other sources of protein, stating the different behavior of the proteins depending their nature. These authors associated this behavior to the heat-induced gelation capacity of whey and egg proteins. Soy proteins also form heat-induced gel structures, which get stiffer after cooling (Bainy, Tosh, Corredig, Woodrow, & Poysa, 2008), that could explain the high viscosity of β CC batters despite the replacement of rice starch. When breakdown was compared, higher values were observed for batters containing added proteins, thus batters' stability during cooking was lowered with the proteins. Conversely, the setback values were only significantly modified when β CC was present in the batter, likely, this protein favored the amylose retrogradation.

The RVA curves (Fig. 1) for the batters showed plots similar to native rice flour pasting pattern. This pattern consists in an increment in viscosity to reach a peak viscosity during

heating and breakdown on holding at 95 °C followed by setback during cooling (Heo et al., 2014). However, even when the same trend was obtained for all batters, irregular lines and multiple peaks before and after the peak viscosity were observed especially in RVA curves from rice flour (Fig. 1). These irregularities were attributed to destabilization of the batter during the heating stage. When proteins were blended with rice flour, the shape of the RVA curves became more regular, especially in gluten batters. The vanishing of irregular peaks in RVA curves of batters that contained gluten and β CC could be associated to a higher water absorption of the proteins in comparison to the rice flour components leading to the thickening of the suspension and thus to less destabilization. The higher stability in the RVA curves of β CC and gluten batters could also be associated to the formation of network structures during heating among proteins and rice flour components. Matos et al. (2014) associated the changes in the rheological behavior during heating of gluten-free batters supplemented with proteins, to the formation of a more rigid protein network promoted by the protein denaturation compared to the rice-based control. On the other hand, the small irregular peaks in batters containing β CC could be related to the dissociation of proteins due to the heating treatment causing protein aggregation (Bainy, Corredig, Poysa, Woodrow, & Tosh, 2010; Matos et al., 2014). The aggregation of soybean proteins at temperatures lower than 95°C where the peak viscosity of the rice flour is obtained has been reported before (Bainy et al., 2010; Marco & Rosell, 2008a).

3.2 Bread characterization

The effect of the replacement of rice flour with 10% proteins in the quality of breads was evaluated. The crumb color, textural parameters, 2D area and cell structure features are summarized in Table 2. The color of the crumb was affected by the addition of proteins.

Breads that contained β CC or wheat gluten presented lower lightness and higher yellowness compared to rice flour counterparts. The differences in breads color were associated to the color of the proteins powders. Gluten ($L^*=85.9 \pm 0.3$, $a^*=-0.4 \pm 0.0$, $b^*=13.8 \pm 0.4$) and β CC ($L^*=88.3 \pm 0.1$, $a^*=-1.2 \pm 0.0$, $b^*=14.1 \pm 0.6$) used herein had lower L^* and higher b^* values compared to the values for rice flour ($L^*=92.8 \pm 0.1$, $a^*=-1.0 \pm 0.0$, $b^*=5.4 \pm 0.1$). Previous reports have shown the same behavior in the color of breads produced with rice flour and supplemented with proteins and associated these changes to the color of raw materials (Marco & Rosell, 2008a; Shin et al., 2010). Ziobro, Juszczak, Witczak, & Korus (2016) also found that the addition of proteins modified the color of gluten-free breads by increasing the darkening and yellowness of crumbs. Moreover, according to these authors, the color changes were advantageous resulting in higher organoleptic acceptability by consumers. The a^* parameter presented values ranging from -1.53 to -1.11. These values were similar to those reported for rice flour breads (Garzon et al., 2017). The differences in the color of the raw materials compared to breads were associated to the baking process which enhanced non-enzymatic browning reactions, favored by the supplemented proteins (Marco & Rosell, 2008a).

Hardness and chewiness were increased due to the addition of gluten and β CC proteins (Table 2). Previous reports also found an increment in the crumb hardness caused by the addition of proteins to rice flour formulations (Marco & Rosell, 2008a; Storck et al., 2013). Although no significant differences were detected, breads containing β CC presented the highest hardness values. This could be associated to the low water binding capacity of the rice flour/ β CC bread formulation (Table 1). Aprodu et al., (2016) found a negative relationship between the water absorption and the hardness of the crumb of gluten-free

277 breads. Although, it must be stressed that the increment in the crumb hardness of breads
278 that contained gluten or β CC could be also attributed to the formation of more rigid
279 structures among the supplemented proteins and rice flour components during the thermal
280 treatment of baking.

281 Interestingly, bread springiness, which is positively related to crumb elasticity, increased in
282 breads that contained gluten or β CC (Table 2). Cohesiveness and resilience also increased
283 due to the protein addition, but compared to rice flour breads, the increment was only
284 significant when the supplemented protein was wheat gluten. High values of cohesiveness,
285 springiness and resilience are desirable since they represent lower disaggregation during
286 mastication and adequate crumb elasticity (Cornejo & Rosell, 2015; De La Hera, Rosell, &
287 Gomez, 2014). It must be remarked that no significant differences were found in these
288 texture parameters when breads containing β CC were compared to those containing vital
289 gluten. Moreover, β CC values were higher than those obtained for gluten-free formulations
290 that used other non-gluten proteins (Krupa-Kozak, Baczek, & Rosell, 2013; Marco &
291 Rosell, 2008a; Shin et al., 2010; Storck et al., 2013) and most of the commercial gluten-free
292 breads analyzed by Matos and Rosell (2012).

293 The 2D area and crumb cell features were evaluated since they are related to the CO₂
294 binding ability during both proofing and baking. Breads produced with 10% β CC had the
295 highest 2D area (Table 2). This is consistent with a previous report where β CC and corn
296 starch were blended to produce model breads which showed higher 2D area in comparison
297 to gluten-corn starch (Espinosa-Ramírez et al., 2018). Authors attributed this significant
298 improvement to higher retention of carbon dioxide by β CC proteins during proofing. The
299 cell density, which represents the number of cells in one cm², presented lower values when

proteins were blended with rice flour, being significant in β CC breads (Table 2). Likewise, β CC breads presented the highest mean cell areas among formulations. Ziobro et al. (2016) also found that the addition of non-gluten proteins to gluten-free bread formulations resulted in crumbs with lower cell density and in most cases, higher cell or loci sizes. Marco and Rosell (2008a) found that the formation of networks during heating in gluten-free formulations was favored by the addition of proteins and hydrocolloids. This network gave strength to the expanding cells of the dough and led to an improved crumb structure with less number and larger size of gas cells (Marco & Rosell, 2008a). In the present study no hydrocolloids were used. Therefore, the results support the assumption that β CC forms a strong network able to retain the gas released during proofing. The cells shape was similar for all breads, as the cell circularity did not show significant differences among formulations (Table 2).

3.3 Protein quality of breads

The protein content, amino acid profile, PDCAAS and IVPD of breads are depicted in Table 3 and Fig. 2. Breads produced with composite flours containing β CC or gluten had almost twice the protein content of the rice flour breads. As expected, breads supplemented with β CC contained the highest protein content because the β CC contained higher protein purity compared to the vital wheat gluten. Matos-Segura and Rosell (2011) analyzed eleven commercial gluten-free breads and found a protein content ranging from 0.9 to 15.1 g/100 g (dry basis, db) with a mean value of 4.8 g/100 g. The protein content reported for white wheat breads was around 13 g/100 g (db) (Villarino et al., 2015). Therefore, the bread

produced with a composite flour containing 10% β CC and rice flour contained higher protein content compared to commercial gluten-free breads and wheat breads.

The amino acid profiles of breads are presented in Table 3. Breads containing gluten presented up to 99% and 50% more proline and glutamic acid + glutamine, respectively, compared to the other bread formulations. In contrast, gluten breads had the lower concentrations of the amino acids: aspartic acid + asparagine, arginine and lysine, among breads. This is consistent with the amino acid profile of gluten proteins, which is characterized by high levels of proline and glutamine and low contents of charged amino acids (Wieser, 2007). The unique amino acid composition and its sequence determine the type of inter- and intra-chain interactions which promote the molecular weight distribution of gluten fractions, especially its polymeric nature, which is recognized as a determinant factor of dough properties and baking performance (Veraverbeke & Delcour, 2002). On the other hand, the amino acids that had a similar content in both gluten and β CC breads were alanine, glycine, threonine and valine. In contrast, 100% rice flour breads presented higher concentrations of these amino acids. The non-covalent interactions formed by the agglomeration of hydrophobic amino acids or electrostatic interactions and hydrogen bonds are necessary to stabilize the gluten network (Ortolan and Steel, 2017). The amino acids that were similar among gluten and β CC formulations but were different to rice breads, could be related to important non-covalent interactions that lead to net-like structures. In this backdrop, the concentration of these amino acids would be essential to favor those net-forming interactions. Breads containing 10% β CC had the lowest content of sulfur amino acids among breads (Table 3) since soybean proteins have low concentrations of these sulfur-containing amino acids (Karr-Lilienthal, Grieshop, Spears, & Fahey, 2005).

Disulfide bonds formed by the crosslinking of cysteine residues are the most important structural element of the gluten network (Ortolan and Steel, 2017). However, the similar content of cysteine in breads produced with 100% rice flour or substituted with 10% gluten, proves that the presence of this amino acid is not enough to favor these interactions.

To evaluate the protein quality, the limiting amino acid was determined based on the FAO/WHO/ONU requirement of a two-year infant (Table 3). In most instances, lysine is the limiting amino acid in wheat bread and other cereal-based foods (Villarino et al., 2015). However, when rice flour was blended with β CC, the lysine content increased 37% in breads. This led to an increment from 0.83 to 1.14 in the amino acid score of lysine. Lysine was the essential amino acid present in the lowest concentration compared to the requirement for infants. Therefore the limiting amino acid score was calculated for lysine, being rice flour and rice flour + 10% gluten the breads, which did not fulfill the WHO/FAO/ONU (2007) requirements for 1-2 years infants (which covers the range appropriate for human adults) (Table 3). Even when the replacement of rice flour with 10% β CC led to lower levels of cysteine + methionine, the suggested requirement for sulfur amino acids (WHO/FAO/ONU, 2007) was still fulfilled by this composite bread. This proved that the selected level of substitution of rice flour with 10% β CC led to the improvement of the amino acid profile of gluten-free breads based on rice flour.

The digestibility of proteins is an indicative of their amino acids availability (Hsu et al., 1977). The IVPD of breads containing 10% gluten was the highest among bread formulations (Fig. 2). Villarino et al. (2015) found a lower IVPD value for wheat bread (78.0%) compared to that obtained herein for breads containing wheat gluten (Fig. 2). The higher IVPD could be associated to a lower interaction between gluten proteins and rice

starch in comparison to those occurring with wheat starch, which led to a higher accessibility of these proteins to hydrolysis. On the other hand, the addition of 10% β CC did not induce significant differences in the IVPD values compared to 100% rice flour breads (Fig. 2). However, β CC proteins seemed to be more rapidly accessible to proteases since the drop of pH, which is related to the release of the amino acids carboxyl groups (Hsu et al., 1977), was faster for β CC breads in comparison to rice flour proteins (Fig. 2). The IVPD values for β CC gluten-free breads were comparable to those obtained for wheat bread (Villarino et al., 2015) and rice flour gluten-free cakes supplemented with legumes (Gularte, Gómez, & Rosell, 2012).

The PDCAAS is a quality index for food protein sources that considers their digestibility and limiting amino acid score (WHO/FAO/UNU, 2007). In the present study, the scores for lysine were used for the PDCAAS calculation (Table 3). Breads containing β CC presented the highest PDCAAS value, which was 20% higher compared to the 100% rice flour breads. The improvement in the PDCAAS was associated to the complementary effect of the rice proteins and the β CC rich in lysine as well as its adequate IVPD. Moreover, PDCAAS of β CC breads was 3.4 times higher than that reported by Villarino et al. (2015) for wheat bread. This proved that the substitution with 10% β CC led to the improvement of the protein quality of rice flour gluten-free formulations.

3.4 Microstructure of doughs and breads

Scanning electron microscopy was used to evaluate the effect of the addition of β CC on the microstructure of fermented doughs and breads (Fig. 3). A reduction of pore geometry was

observed from dough to bread microstructure, shifting to smaller closing size, as previously reported by Bajd and Sersa (2011), when following the pore size dependency with proofing and baking of doughs and breads obtained from different mixed flours. Micrograph of the 100% rice flour fermented dough showed an amorphous structure where starch granules were held together by the proteins but no gas cells were observed (Fig. 3A). On the other hand, the structure of fermented doughs containing gluten or β CC was significantly different. These doughs presented a continuous protein network, where gas cells were trapped covered by a veil of rice starch granules (Fig 3B-C). Compared to gluten, a higher size of gas cells in β CC doughs and also more uniform distribution of the starch granules over the entire protein network was observed.

In bread micrographs, a continuous structure resulted from gelatinized starch and denatured proteins in which air cells were visible (Fig. 3D-F). Therefore, the use of the water binding capacity as an indicator of the water required for forming the gluten free structure allowed an adequate interaction of the components after heating. However, several differences were noticed in the microstructure depending on the proteins added. For 100% rice flour breads continuous zones with no air cells were observed, and wherever pores, those were smaller (Fig. 3D), in comparison to the formulations with proteins. Conversely, β CC and gluten breads presented the same net-like structure, but β CC displayed higher number of larger pores and thinner lamellae. Previous reports highlighted the importance of the addition of proteins to gluten-free formulations to form web-like structures and stabilize gas cells (Ahlborn, Pike, Hendrix, Hess, & Huber, 2005; Marco & Rosell, 2008a). However, these authors used several additives including hydrocolloids and crosslinking enzymes to obtain improved aerated structures similar to those produced in wheat bread. The breads produced

herein had lean formulations and no hydrocolloids/enzymes were used. Notwithstanding, β CC breads produced a web-like structure similar to those produced with gluten-rice flour and to wheat bread (Ahlborn et al., 2005).

The development of the net-like structures observed in SEM images after breadmaking could be the reason of the increased hardness and chewiness observed in breads containing wheat vital gluten or β CC, but also of their improved values of springiness, cohesiveness and resilience (Table 2). Moreover, these results confirmed the high capacity of β CC proteins to give the strength needed to hold the expanding CO₂ during fermentation and baking, which led to the observed higher 2D bread crumb area and mean cell areas (Table 2). Therefore, β CC serves as structuring agent and has the potential to form net-like structures similar to gluten during breadmaking.

4. Conclusion

Rice-based yeast-leavened breads with adequate features (volume and texture) were produced from batters using the water binding capacity of the flours as the parameter to set the optimum amount of water. β CC (10%) when incorporated to rice flour for the production of gluten-free bread provided the required network for holding the carbon dioxide during proofing and also for allowing expansion during baking. In fact, SEM micrographs reveal that β CC brought about a net matrix similar to the one obtained in the presence of wheat gluten, and without the addition of any hydrocolloid. Breads containing 10% β CC showed improved quality in terms of color, texture, 2D area, crumb structure and protein quality in comparison to the bread counterpart elaborated with 100% rice flour. It is

noteworthy to remark that a concentration of 10% β CC allowed to reach a complete balance of essential amino acids in rice flour formulations. Overall, the use of this ingredient is useful to fulfill both technological and nutritional challenges in gluten-free breads.

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580 **Table 1. Effect of gluten and β CC (10% flour replacement) on the rice flour hydration and batter properties^a**

Parameter	Formulation					
	100% Rice flour		90% Rice flour + 10% Gluten		90% Rice flour + 10% β CC	
WBC (g water/100 g flour)	134 $\pm$	2 a	130 $\pm$	2 a	121 $\pm$	5 b
<i>Pasting properties</i>						
Onset pasting temperature ($^{\circ}$ C)	67.3 $\pm$	0.7 a	68.1 $\pm$	0.5 a	66.4 $\pm$	0.5 a
Peak viscosity (mPa)	2221 $\pm$	16 c	2839 $\pm$	175 a	2554 $\pm$	81 b
Breakdown (mPa)	1323 $\pm$	1 b	2062 $\pm$	185 a	1936 $\pm$	89 a
Final viscosity (mPa)	1462 $\pm$	7 b	1333 $\pm$	4 c	1747 $\pm$	11 a
Setback (mPa)	564 $\pm$	8 b	556 $\pm$	14 b	849 $\pm$	1 a

581 ^aValues are means and standard deviation of at least two replicates; means with different letters within the same parameter differ
582 significantly ($P < 0.05$). WBC= Water binding capacity; β CC= β -conglycinin concentrate; mPa= millipascal.

583

584

585 **Table 2. Effect of the substitution of gluten and β CC in the color, texture, 2D area and crumb structure features of yeast-**
586 **leavened rice flour breads^a**

Parameter	Formulation					
	100% Rice flour		90% Rice flour + 10% Gluten		90% Rice flour + 10% β CC	
<i>Color</i>						
<i>L*</i>	79.2 $\pm$ 1.2	a	72.5 $\pm$ 2.1	b	72.9 $\pm$ 1.7	b
<i>a*</i>	-1.37 $\pm$ 0.03	ab	-1.11 $\pm$ 0.29	a	-1.53 $\pm$ 0.18	b
<i>b*</i>	7.8 $\pm$ 0.5	c	13.7 $\pm$ 0.8	a	10.1 $\pm$ 1.2	b
<i>Texture</i>						
Hardness (g)	96.0 $\pm$ 5.0	b	126.8 $\pm$ 22.0	a	138.2 $\pm$ 15.7	a
Springiness	0.95 $\pm$ 0.06	b	1.00 $\pm$ 0.00	a	1.00 $\pm$ 0.01	a
Cohesiveness	0.68 $\pm$ 0.04	b	0.75 $\pm$ 0.06	a	0.71 $\pm$ 0.03	ab
Chewiness (g)	61.6 $\pm$ 6.4	b	94.0 $\pm$ 12.8	a	98.3 $\pm$ 8.3	a
Resilience	0.34 $\pm$ 0.04	b	0.39 $\pm$ 0.03	a	0.37 $\pm$ 0.02	ab
2D Area (cm ²)	2.98 $\pm$ 0.23	b	2.88 $\pm$ 0.21	b	3.37 $\pm$ 0.26	a
<i>Crumb cell structure</i>						
Cell density (u/cm ²)	13 $\pm$ 2	a	12 $\pm$ 1	ab	11 $\pm$ 2	b
Mean cell area (mm ²)	1.99 $\pm$ 0.92	b	2.31 $\pm$ 0.44	b	3.12 $\pm$ 0.64	a
Cell circularity	0.37 $\pm$ 0.04	a	0.36 $\pm$ 0.03	a	0.39 $\pm$ 0.04	a

587 ^a Values are means and standard deviations of at least three replicates; means with different letters within the same parameter differ
588 significantly ($P < 0.05$). β CC= β -conglycinin concentrate.

589

Table 3. Effect of the substitution of gluten and β CC in the protein quality characteristics of rice flour yeast-leavened breads^a

Parameter	Formulation										WHO/FAO/ONU suggested requirement for essential amino acids ^c		
	100% Rice flour				90% Rice flour + 10% Gluten				90% Rice flour + 10% βCC				
Protein (% db) ^b	7.81	±	0.15	c	15.31	±	0.11	b	16.10	±	0.15	a	
<i>Amino acid content (g/100 g protein)</i>													
Alanine	5.78				4.45				4.66				
Arginine	8.06				5.70				8.40				
Aspartic Acid + Asparagine	9.14				6.01				10.78				
Glutamic Acid + Glutamine	17.88				26.83				20.36				
Glycine	4.70				3.97				3.79				
Histidine	2.42				2.26				2.42				1.8
Isoleucine	4.44				4.15				4.77				3.1
Leucine	8.60				7.73				8.24				6.3
Lysine	4.30				2.99				5.91				5.2
Methionine + Cysteine	2.82	+	2.42		2.15	+	2.27		1.72	+	1.52		2.6
Phenylalanine + Tyrosine	5.65	+	3.23		5.51	+	3.41		5.85	+	3.31		4.6
Proline	4.30				8.57				4.74				
Serine	4.84				4.25				4.36				
Threonine	3.63				3.05				2.96				2.7
Tryptophan	1.08				1.06				0.85				0.74
Valine	6.32				5.19				5.10				4.2
Limiting amino acid score	0.83				0.57				1.14 ^d				
PDCAAS	(lys)				(lys)								
	0.65				0.47				0.78				

590 ^a β CC= β -conglycinin concentrate; PDCAAS= Protein digestibility corrected amino acid score.

591 ^b Values are means and standard deviations of three replicates; means with different letters within the same parameter differ significantly ($P <$
592 0.05).
593 ^c Suggested requirements for 1-2 years infants. Values are expressed in g/100 g protein.
594 ^d Value was truncated to 1.0 for the calculation of PDCAAS as recommended by WHO/FAO/ONU, 2007.

595

596 **Fig 1. Viscosity profile determined by Rapid Visco Analyser of batters produced with**
 597 **100% rice flour (black line); 90% rice flour + 10% gluten (dotted line); and 90% rice**
 598 **flour + 10% β -conglycinin concentrate (gray line).** Dashed line represents the
 599 temperature profile. Values are means of two replicates.

Fig. 2 *In vitro* protein digestibility (IVPD) and pH drop in yeast leavened breads produced with 100% rice flour (□); 90% rice flour + 10% gluten (○); and 90% rice flour + 10% β-conglycinin concentrate (×).

Fig 3. Scanning electron microscope images of the fermented doughs (A-C) and yeast-leavened breads (D-F) of formulations with 100% rice flour (A and D), 90% rice flour + 10% gluten (B and E), and 90% rice flour + 10% β -conglycinin concentrate (C and F) at 700x magnification.