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Bacterial nanocellulose as a potential additive for wheat bread

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ABSTRACT

Bacterial nanocellulose (BNC) is an emerging nanomaterial with a morphologic structure of a 3-D network and unique properties produced by several species of bacteria. The objective of the present work was to evaluate whether the addition of BNC improved the baking quality of wheat flours, making a change in the viscoelastic behavior of the mass. A study of the rheological behavior of wheat bread dough containing BNC was performed by thermo-rheological and isothermal dynamic oscillatory experiments. The baking response and bread quality parameters were also analyzed. BNC increased specific volume, and moisture retention, decreasing browning index. Although BNC produced both raw and heat-treated doughs with more elastic characteristics, textural studies revealed that the addition of BNC reduced firmness of bread crumb. Confocal laser scanning microscopy observations showed differences in gluten filaments between control and BNC crumb samples that could explain the larger average porous size of BNC crumb. BNC could be used as improver in the bread-making performance.

1. Introduction

Bread and other fermented products have remained a staple food for thousands of years. In the past the key role for bread was the simple provision of energy, but in more recent years the role that wheat-based products play in delivering additional nutritional benefits has become more prominent. Such benefits include the provision of fiber and essential nutrients beyond the simple value of protein and carbohydrate.

Nowadays, there is a growing demand for a new generation of healthier food products, which, at the same time, are required to have excellent sensory quality (Escalada Pla, Rojas, & Gerschenson, 2013). The use of additives like oxidants, enzymes, emulsifiers and hydrocolloids is a common practice to improve breadmaking performance, to facilitate processing, to compensate for variations in raw materials, to guarantee constant quality, and to preserve freshness and food properties. In general, addition of hydrocolloids to dough has important consequences on breadmaking procedure: they require a supplementary addition of water and the interactions they establish with the other dough components lead to changes in rheological properties of dough. These changes can also vary the sensorial attributes of the final product, resulting in an impact on consumers' acceptability (Gómez, Ronda, Caballero, Blanco, & Rosell, 2007). The effect of hydrocolloids varies depending on their chemical structure and on the specific property that is being studied (Guarda, Rosell, Benedito, & Galotto, 2004; Rosell, Rojas, & Benedito de Barber, 2001). Bread can be enriched with dietary fiber, including wheat, gums, such as guar gum and modified celluloses, and β-glucans. However, the addition of fiber to dough is a subject of controversy in the literature. On one hand it may cause: reduction of loaf volume, increase of crumb firmness, and dark crumb appearance (Wang, Rosell, & Benedito de Barbera, 2002). On the other hand, they increase the total dietary fiber intake of the consumer, and decrease the caloric density of baked breads (Stauffer, 1990). It has also been informed that the use of high methoxyl pectin produces higher specific volume and softened bread crumb (Ponzio, Ferrero, & Puppo, 2013). Clearly, fiber in dough interacts with the gluten matrix. To what extent, which types of fiber and chain lengths, and whether it has mainly positive or negative effects on the quality varies depending on the chemical structure of the hydrocolloids added (Eckardt et al., 2013).

Particles in the nanometer-sized range can often be produced using food-grade biopolymers such as proteins or polysaccharides (Ritzoulis, Scoutaris, Papademetriou, Stavroulias, & Panayiotou,

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2005). In particular, BNC—also called bacterial cellulose, microbial cellulose, or biocellulose—is a biopolymer with a morphologic structure of a 3-D network formed by several species of aerobic bacteria, the most efficient being *Gluconacetobacter* spp., as a pure component of their biofilms (Klemm et al., 2006). BNC is formed as a polymer and nanomaterial by biotechnological assembly processes from low-molecular weight carbon sources, such as D-glucose. BNC is excreted as exopolysaccharide at the interface to the air as a ribbon-shaped fibrils, less than 100 nm wide, which are composed of much finer 2–4 nm nanofibrils (Brown & Laborie, 2007) The resulting form-stable BNC hydrogel is composed of an ultrafine nanofiber network structure enclosing up to 99% water (Cerrutti et al., 2016; Gama, Gatenholm, & Klemm, 2013; Robson, 2013; Tabuchi, 2007; Vázquez, Foresti, Cerrutti, & Galvagno, 2013). This fine structure makes BNC different from other microbial polysaccharides, producing high water holding capacity, high tensile strength, high purity, and flexibility (Iguchi, Yamanaka, & Budhiono, 2000).

BNC is regulatory classified as "generally recognized as safe" (GRAS) and was accepted as such by the USA Food and Drug Administration in 1992 (Shi, Zhang, Phillips, & Yang, 2014; Strom, Ohgren, & Ankerfors, 2013) It may be used as a thickening, stabilizing, gelling, or suspending agent to produce a variety of foods such as desserts, Tofu, ice cream, and chocolate drinks, (Shi et al., 2014). It is regarded as an acceptable fat mimic in the production of emulsified meat products (Lin & Lin, 2004; Lin, Chen, & Chen, 2011)

The aim of the present research was to determine the potential use of bacterial nanocellulose as an additive in bread-making through a systematic study on its influence on the rheological properties of bread doughs and the final quality of the resulting breads. The effect of storage at room temperature on staling of baked bread with and without BNC was also analyzed.

2. Materials and methods

2.1. Bacterial nanocellulose production

BNC pellicles were produced by *Gluconacetobacter xylinus* NRRL B-42 gently provided by Dr. Luis Ielpi (Fundación Instituto Leloir, Buenos Aires, Argentina) following the protocol of Vázquez et al. (2013).

Inoculate were cultured for 48 h in Erlenmeyer flasks containing Hestrin and Schramm (HS) medium (%, w/v): glucose, 2.0; peptone, 0.5; yeast extract, 0.5; anhydrous disodium phosphate, 0.27; citric acid, 0.115. The pH was adjusted to 6.0 with dil. HCl or NaOH. Agitation was provided by an orbital shaker.

For BNC production static incubation were performed at 28 ± 1 °C for 14 days in Erlenmeyer flasks containing HS medium modified by replacing D-glucose by glycerol and a ratio "volume flask: volume medium" of 5:1 was maintained. The initial pH of production media was 5.0. All media were sterilized by autoclaving (121 °C, 15 min).

Afterwards BNC pellicles were rinsed with water to remove the culture medium, and then boiled in 2% w/v NaOH solution for 1 h in order to eliminate the bacterial cells from the cellulose matrix. Then, pellicles were washed with distilled water till neutralization (Moosavi-Nasab & Yousefi, 2011). All the reagents used were analytical grade.

2.2. Dough preparation

Commercial Argentine wheat flour provided by Molinos Campodónico S.A. (La Plata, Argentina). Flour composition was: protein 13.26 ± 0.03 g/100 g (Kjeldahl factor = 5.7), moisture 13.07 g/100 g, lipids 1.2 ± 0.1 g/100 g and ash 0.68 ± 0.04 g/100 g, dry gluten values were 9.01 ± 0.11 (g gluten/100 g flour), as informed by the producer.

Basic dough formula (Strom et al., 2013) consisted in: wheat flour (100 g), dry yeast (1.0 g), sugar (0.9 g), NaCl (2.6 g), and total water (54.1 g), this formulation was considered as a control. Besides a second formulation with 0.14 g of BNC (dry-basis) was prepared.

As BNC was obtained in gel form, forming a pellicle several mm thick, it was first dispersed in water and processed in a blender (Winco w-1902, Guangzhou Hongpai Household Electric Appliances Co., Guangdong, China) during 2.5 min at low speed and 2.5 min at high speed, before adding the dispersion (0.25 g dry BNC/100 g water) to the rest of the components. BNC pellicle water content has to be previously determined by drying at 105 °C until constant weight to evaluate the amount of water incorporated to the dough in the BNC gel.

After the breads were baked, specific volume, yield, and textural properties (TPA, Instron Universal Testing Machine 5982, Instron Corp., Norwood, USA) were measured initially and during storage at 20 °C, in order to establish whether the parameters were influenced by the added BNC.

2.3. Breadmaking

Ingredients were mixed (Spar Mixer SP502, SPAR Food Machinery MFG Co., Ta-li, Taiwan) for 5 min, rested for 15 min, kneaded and divided into balls (70 g), molded into silicone muffin cups, rested again for 15 min and finally proofed at 30 °C in a drying oven (Taisite 101-1AB, Tianjin Taisite Instrument Co., Tianjin, China). After proofing baking was conducted at 195 °C. After 23 min the breads were withdrawn from the oven, turned out of the molds and placed in ambient air to cool for 2 h before further analyses.

2.4. Bread storage

One batch was prepared from each formulation (control and BNC), and divided in 4 portions, one was initially analyzed for crumb moisture and texture (2.5.2 and 2.5.3.) and the rest was analyzed daily during 3 days for study bread staling.

2.5. Bread characterization

2.5.1. Specific volume, crumb moisture, and yield

Bread volume was determined by seed displacement in a loaf volume meter. Ten replicates were performed for each formulation. Crumb moisture was performed on four loaves from each batch and determined following AACCI 44-19 (AACCIM, 2014). Yield was determined by weighting the loaves before and after baking (4 loaves per batch) and was expressed as g baked bread/100 g dough.

2.5.2. Crumb texture

Ten cylindrical samples of 2.5 cm diameter and 1 cm height were obtained from bread crumb. Texture parameters were evaluated using a texture analyzer (Instron Universal Testing Machine 5982, Instron Ltd., MA, USA). Sample was submitted to two cycles of compression up to 40% of the original height with a cylindrical probe (diameter 5 cm). Force time curves were obtained at a crosshead speed of 0.5 mm/s. Product hardness (firmness), adhesiveness, elasticity, cohesiveness, and resilience were determined.

2.6. Rheological analyses of the dough

Non-yeasted doughs were prepared for rheological measurements in an oscillatory controlled stress rheometer (RS600, Haake RS600, Thermoelectron, Germany) provided with a temperature

control unit (K-15 Haake, Thermoelectron, Germany) at 25 °C. Measurements were performed in using a serrated plate-plate sensor system (35 mm) with a gap of 1 mm between plates. In order to relax the samples before the measurements, all samples were allowed to rest for 15 min. Samples perimeters were covered with a thin film of silicone oil and the measuring system was covered with a special device to prevent was used to prevent evaporation of the exposed edge of the during testing.

Data were processed using the IRIS Rheo-hub 2011 program (IRIS Development LCC., Amherst, MA) to perform the spectra calculations (Winter & Mours, 2006).

2.6.1. Dynamic oscillatory measurements

Stress sweep tests (25 and at 80 °C) at a fixed frequency (6.28 rad/s - 1 Hz) were made to determine the linear viscoelastic region(LVR) of all samples; from this analysis a stress value of 10 Pa was chosen for all the frequency tests.

Frequency sweeps (0.31–628 rad/s) were conducted within the LVR (10 Pa) at 25 °C. Dependence of the storage (G′) and loss (G″) moduli with frequency was obtained in all cases. Small amplitude oscillatory shear analysis was conducted in triplicate for each formulation.

2.6.2. Thermo-rheological assays

After equilibration at 25 °C, the samples were sheared at a fixed frequency of 6.28 rad/s with a stress of 10 Pa during all the thermal ramps and isothermal process. The thermal scanning started with an isothermal stage at 25 °C for 3 min, followed by heating to 85 °C (heating rate 5 °C/min). After this heating stage the sample was held isothermally at 85 °C for 3 min. Then, a cooling stage was done from 85 °C to 25 °C at 5 °C/min. Lastly an isothermal step at 25 °C was performed for 5 min. Changes in the dynamic storage modulus, G′ (Pa), loss modulus, G″, and loss tangent (δ) were monitored continuously throughout the simulated gelling process at 6.28 rad/s (1 Hz) frequency. All measurements were performed within the linear viscoelastic range which has been previously determined at 25 °C and 85 °C. Thermo-rheograms presented correspond to mean values of two replicates per formulation.

2.7. Image analysis

2.7.1. Crust browning measurement

To evaluate changes in the surface browning of the baked loaves images of bread crust were captured using a flatbed scanner (HP OfficeJet 4500, Hewlett Packard, USA). Once data acquisition was done, image processing was performed. (Brosnan & Sun, 2004; Purlis & Salvadori, 2009).

Twelve replicates for each formulation were considered from different positions in the baking oven to avoid this effect on the color variation. Since digital images are acquired in the RGB color space, those values were converted to the XYZ tristimulus values (Du, Cheng, & Sun, 2012; Mendoza, Dejmek, & Aguilera, 2006):

Once the color conversion was done, the browning index (BI) was calculated by applying the equation defined by Buera, Lozano, and Petriella (1986):

$$BI = \frac{(x - 0.31)}{0.172 \cdot 100} \quad (1)$$

were x is the chromaticity coordinate calculated from the XYZ values according to: $x = X/(X + Y + Z)$. The BI represents the purity of brown color when non-enzymatic browning takes place. Although this index was originally developed to represent browning of liquid model systems, recently, it has been satisfactorily used to report browning variation of several bakery products (Ureta, Olivera, & Salvadori, 2014; Yang et al., 2014).

2.7.2. Crumb structure characterization

Digital image analysis was used to characterize the crumb structure. Images of bread crumb were captured using a flatbed scanner (HP OfficeJet 4500, Hewlett Packard, USA). The scanned image (300 dpi) was analyzed using the Image J software (NIH, available at http://rsb.info.nih.gov/ij/), that uses the contrast between the two phases (pores and solid part) in the image. Eight digital images were processed for each batch. The scanned color image was first converted to gray scale and cell area/total area (cm^2/cm^2), average pore diameter, and number pores were measured. Equivalent volume mean (D[4,3]), which is identical to the weight equivalent mean if density is constant was calculated as:

$$D[4,3] = \frac{\sum_{i=1}^{n} D_j^4}{\sum_{i=1}^{n} D_j^3} \quad (2)$$

2.7.3. Confocal laser scanning microscopy (CLSM)

2.7.3.1. Sample preparation. A mixture of rhodamine B (0.0001%) and fluorescein isothiocyanate (FITC) (0.001%) in distilled water was used for non-covalent labeling. A small portion of dough was cut and then spread on a glass slide with a rolling pin; immediately it was imbibed with the dye solution. The sample was let to rest for an hour within a closed recipient and in darkness, and then the specimen was washed with distilled water and covered with a glass cover slip. Dough samples did not show autofluorescence.

2.7.3.2. Confocal microscopy system. A confocal laser scanning microscope (Olympus FV 300/BX61, Tokyo, Japan) equipped with diode and HeNe lasers was used. The excitation wavelengths were 488 nm (FITC) and 543 nm (rhodamine B) and the emission wavelengths were 525 nm (FITC) and 625 nm (rhodamine B). At least ten photographs (5 by each replicate) with the same magnification were obtained from representative fields. Floview Application software and Image J 1.43 were employed in the image analysis. Each micrograph was RGB color split and then was corrected by shading applying FFT filtering (Walter, 2003). The corrected image was subjected to an automatic thresholding and converted in a binary image as described by Peighambardoust, van der Goot, van Vliet, Hamer, and Boom (2006).

2.8. Statistical analysis

Each experiment was replicated at least three times (three or more independent batches were baked with 12 loaves per batch). The software SYSTAT (SYSTAT, Inc., Evanston, IL) was used to perform all statistical procedures like analysis and variance, t-tests, and simultaneous pairwise comparisons (least significance differences, LSD). Differences in means and F-tests were considered significant when $P < 0.05$. Experimental data was reported as mean values and standard error of the mean (SEM) between parentheses.

3. Results and discussion

3.1. Effect of BNC on bread quality

While there are as many opinions on what makes ‘good’ bread as there are bakers and consumers, it is true to say that certain quality characteristics are required for individual bread varieties to be acceptable to the widest cross-section of consumers. However, there are certain features that are usually looked upon when

referring to quality: crust color, crumb structure, loaf volume, texture, and flavor. The first four parameters are easily measured by objective test and were the ones considered in this work.

Product yield is an important parameter to the baker; in this work there were no significant differences between product yield of BNC and control loaves (86.6 g/100g dough and 86.7 g/100g dough, respectively), however Table 1 shows that the addition of BNC produced an increase in loaf specific volume and crumb moisture content. BNC nanofibrils have a large surface area and a highly hydrophilic nature (high quantity of OH at the surface) that makes them a very strong hydrogel which probably contributes to water retention (Gama et al., 2013).

The increase in specific volume is consistent with the changes in crumb porosity observed. Among the different physical properties which can be considered as characterizing bread, porosity is important not only for the mechanical properties of the crumb but also for moisture transfer within the product. Representative images of the breads obtained showed that larger cells appeared in breads prepared with bacterial nanocellulose when compared to the control formulation. Digital image analysis revealed significant differences in terms of crumb structure between both formulations ($P < 0.05$). A significant change in the cell size and distribution was found. Histograms clearly showed that the presence of BNC produced an increase in average pore size (Fig. 1). In this work D[4,3] was 2.45 ± 0.08 for BNC samples, decreasing to 2.16 ± 0.02, for Control bread crumb. It reflects the size of those particles which constitute the bulk of the sample volume. It is most sensitive to the presence of large particulates in the size distribution. The equivalent volume mean (D[4,3]) in leavened baked products indicates that the predominant differences in the crumb structure of bread due to formulation or flour quality is related to the subdivision and coalescence of gas cells, which itself reflects dough strength (Sapirstein, 1999).

The texture of the crumb was evaluated with a Texture Profile Analysis (TPA) (Bourne, 1978) in terms of hardness, cohesiveness, springiness, adhesiveness, and resilience. Crumb softness or firmness is the texture property which has attracted most attention in bread assessment (Cauvain, 2015), recovery or resilience is another property that is usually considered.

Table 1
Product yield, specific volume, and moisture content of BNC and control breads.*

	BNC	Control
Yield (g/100 g dough)	86.6 [a] (0.3)	86.7[a] (0.3)
Specific volume (cm^3/g)	3.04[b] (0.05)	2.63[a] (0.05)
Crumb water content (g/100 g)	41.65[b] (0.07)	40.60[a] (0.04)

*Different superscripts within the same row indicate significant differences according to Tukey's test ($P < 0.05$). Standard error of the mean is given between parentheses.

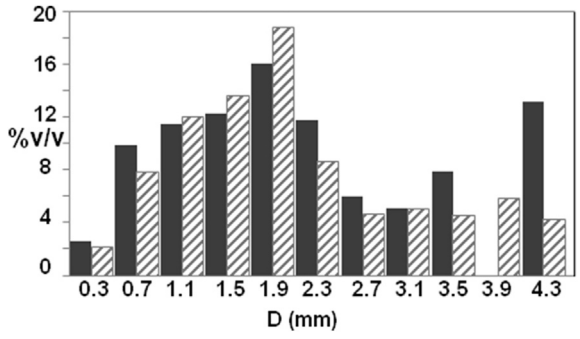

Fig. 1. Cell size distribution of the studied bread formulations expressed as %v/v = 100× cell volume/total volume. ■ Bacterial nanocellulose formulation; ▨ Control.

Table 2
Texture profile analysis (TPA) results and color parameters of BNC and Control bread loaves.*

TPA	BNC	Control
Firmness (N)	2.51 [b] (0.13)	3.31[a] (0.12)
Cohesiveness (J/J)	0.789 [b] (0.003)	0.799[a] (0.003)
Resilience (J/J)	0.44[b] (0.03)	0.48 [a] (0.03)
Color		
L*	55.9[b] (1.0)	51.9[a] (2.1)
a*	7.1[a] (0.2)	7.0[a] (0.3)
b*	14.5[b] (0.4)	17.7[a] (0.8)
Browning index	32.98[b] (0.55)	35.78[a] (0.96)

*Different superscripts within the same row indicate significant differences ($P < 0.05$). Standard error of the mean is given between parentheses.

Under the tested conditions, the addition of BNC previously treated 4 min in a blender resulted beneficial in the characteristics of the baked bread as can be seen in Table 2. A less firm crumb than the control formulation was observed. Still BNC loaves were slightly less resilient and cohesive than the control. There were no differences in springiness and neither formulation showed any measured adhesiveness.

The inclusion of BNC in dough formulation significantly affected crust color. It produces more luminous loaves, with smaller yellowness parameter and browning index than the control breads (Table 2).

3.2. Rheological analysis

3.2.1. Linear viscoelastic region (LVR)

The domain of linear viscoelasticity was established by the oscillatory stress sweep experiment. Fig. 2A shows, as an example, changes in dynamic modulus G* during stress sweeps at a frequency of 1 Hz for both formulations at 25 °C and 85 °C. Storage and loss moduli curves were qualitatively similar, with $G' \gg G''$, all samples presented a relatively large threshold that was independent of the dough composition. As can be seen, the evolution of the dynamic modulus was clearly different at the two temperatures, demonstrating the formation of systems with rather different structural characteristics: from a gel with significant frequency dependence of both moduli within the available frequency range studied at 25 °C (behavior of the dough before gelatinization) to a stronger system at 85 °C (behavior of the dough after gelatinization). Lower frequency dependence and an increase in the viscoelastic constants were observed following heating, indicating a more consolidated system with greater consistency. This behavior was observed in several starch gels and viscoelastic gluten-free doughs (Correa, Añón, Pérez, & Ferrero, 2010; Salvador, Sanz, & Fiszman, 2006).

Although it was clearly observed an increase in both the storage and loss moduli with the addition of BNC, it did not significantly modify the extension of the LVR.

3.2.2. Effect of the addition of BNC on dough rheology

The results of dynamic rheological measurements in the linear viscoelastic range were expressed in terms of the storage modulus (G′) and loss modulus (G″). Results of the dynamic oscillatory tests are presented in Fig. 2B for the two dough formulations; the curves were qualitatively similar for all the formulations assayed. G′ was always greater than G″ in the frequency range measured, and the increase of the two moduli with frequency was small; it corresponds to the characteristics of a weak gel (Ferry, 1980). Several authors have reported a similar trend for flour dough with G′ and G″ increasing with frequency (Agyare, Xiong, Addo, & Akoh, 2004;

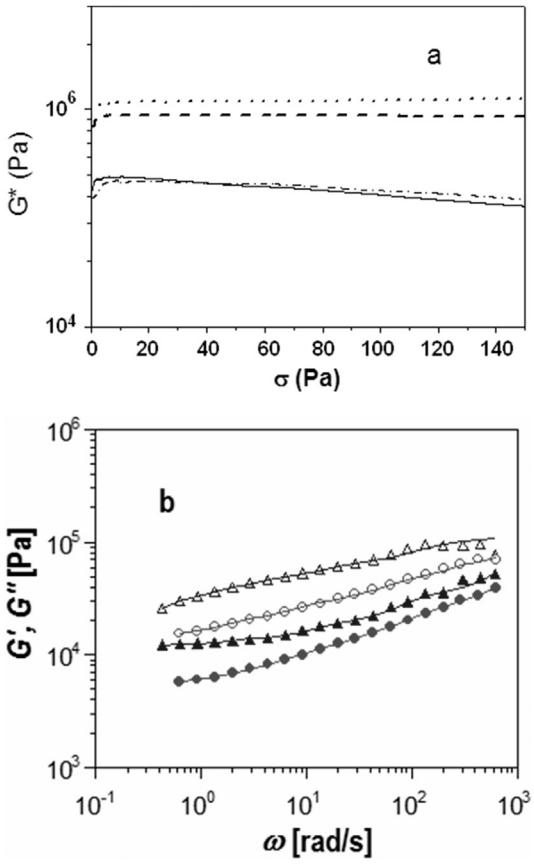

Fig. 2. Rheological analysis: a) Stress sweeps of dynamic modulus G*: —— (BNC, 25 °C), ····· (BNC, 85 °C), —·— (Control, 25 °C), and - - - · (Control, 85 °C). b) Frequency sweep test of unfermented wheat doughs at 25 °C: BNC dough (G′△, G″▲) and control formulation (G′○, G″●). Solid line represents the fitting using Maxwell model.

Dreese, Faubion, & Hoseney, 1988; Kenny, Wehrle, Auty, & Arendt, 2001; Lefebvre, Pruska-Kedzior, Kedzior, & Lavenant, 2003; Lorenzo, Zaritzky, & Califano, 2008; Ribotta, Pérez, León, & Añón, 2004).

The behavior in Fig. 2b could yield to a crossover of both moduli below 10-2 rad/s, indicating that the recovery of the stressed dough network was a slow process and the network was not completely elastic. Dough structure could be visualized as a composite material formed by two phases: a proteinaceous matrix and an inert filled material (starch) (Ravindra, Genovese, Foedgeding, & Rao, 2004). The unique properties of a developed dough, are the result of protein hydration, unfolding, and orientation with complex reactions between sulfhydryl (S—H) and disulfide (S—S) bonds present in gluten. Because BNC is a high molecular weight, hydrophilic molecule it should be expected to interfere (positively or negatively) in gluten development in a way related to its chemical structure; in this work the addition of BNC reinforced the elastic characteristics of the dough. But there is no information about its influence on the structural aspects of gluten network and consequently on dough rheology.

To further quantify these qualitative observations, the methodology proposed by Bruno and Moresi (2005) was applied.It is based on an extension of the polymeric entanglement model proposed by Friedrich and Heymann (1988); assuming that the equilibrium modulus $G_{\infty,\alpha} \ll G'$

Dynamic complex viscosity may be expressed as:

$$\eta^* = \frac{G^*}{\omega} = \frac{\sqrt{G\prime^2 + G''^2}}{\omega} \approx A_\alpha \omega^{(\alpha-1)} \tag{3}$$

$$A_\alpha = \sqrt{\frac{2}{\pi}} * S^*_\alpha \tag{4}$$

Thus, viscoelastic characteristics of doughs could be described in terms of α(order of the relaxation function) and the parameter A_α, which represents a measure of the strength of the cross-linking network. Complex dynamic viscosities were calculated from frequency sweep data and their dependence with frequency was modeled according to Eq. (3).

All the doughs showed similar α values; an average $\alpha \approx 0.21$ was obtained, which reflected the weak dependence on the frequency for both moduli typically observed in gel-like samples (Steffe, 1996). Besides, all the doughs showed a similar shear-thinning behavior over a wide range of frequencies with $\alpha - 1 = -0.79$. A_α changed significantly ($P < 0.05$) with the addition of BNC (from 17,711 to 35,360 Pa s); BNC strengthened the cross-linking network thus resulting in higher A_α values. Under these conditions, the more rigid dough structure (more elastic polymer network) would imply stronger entanglements among hydrocolloids molecules in the composite network.

One of the simplest ways of understanding the linear viscoelasticity is to make use of simple mechanical models. These consist of combinations of linear elastic and viscous elements, i.e. springs and dashpots. A spring is a representation of a linear elastic element that obeys Hooke's law. Similarly, linear viscous response can be modeled using a dashpot. Thus, if a spring and a dashpot are connected in series the simplest representation of a viscoelastic material is obtained, i.e. the so called Maxwell model.

However, experimental data show that the Maxwell model does not account for the stress relaxation behavior of many viscoelastic materials because of their rheological complexity. This problem may be addressed for numerous foods by constructing a model which has several Maxwell elements connected in parallel with a spring. Each of the N Maxwell elements is defined by the elastic response of the spring (G_i) and the relaxation time which is the ratio between the viscosity of the dashpot and the rigidity of the spring ($\lambda_i = \eta_i/G_i$). The behavior of the viscoelastic material is entirely characterized by the knowledge of discrete relaxation spectrum which is represented by the number N and different values of G_i and λ_i. (Ferry, 1980). The following equations are obtained for the storage and loss modulus when the generalized Maxwell model is used to represent the relaxation modulus:

$$G\prime(\omega) = G_e + \sum_{i=1}^{N} G_i \frac{(\omega\lambda_i)^2}{1 + (\omega\lambda_i)^2} \tag{5}$$

$$G''(\omega) = \sum_{i=1}^{N} G_i \frac{(\omega\lambda_i)}{1 + (\omega\lambda_i)^2} \tag{6}$$

As can be observed in Fig. 2B, there was an excellent agreement between the experimental and predicted values obtained using IRIS Rheohub software 2011 (IRIS Development LCC., Amherst, MA, USA), confirming the accuracy of the calculations. Once the relaxation time spectrum was known other material functions such as the plateau modulus G^0_N and the steady-state zero shear rate viscosity (η_0) were evaluated from the discrete relaxation. The plateau modulus is a viscoelastic parameter defined for polymers as the extrapolation of the contribution of the entanglements to the viscoelastic functions at high oscillation frequencies and inversely proportional to the molecular weight between entanglements or

topological constrains (Baumgaertel, De Rosa, Machado, Masse, & Winter, 1991). For the dough containing BNC the obtained parameters were $G_N^0 = 15.3 \times 10^5$ Pa and $\eta_0 = 13.7 \times 10^5$ Pa s, while for the control formulation much smaller values were computed, 7.41×10^5 Pa and 1.48×10^5 Pa s, respectively, showing that BNC definitively reinforces the system.

3.2.3. Temperature sweep test

To analyze the dough behavior during baking a thermo-rheological test was performed on each formulation. Small amplitude oscillatory shear analyses were carried out at a fixed frequency of 6.28 rad/s (1 Hz) with a stress of 10 Pa (L during all the thermal ramps and isothermal process. Changes in the dynamic storage modulus, G′ (Pa), loss modulus, G″, and loss tangent (δ) were monitored continuously throughout the simulated baking process. All measurements were performed within the linear viscoelastic range which has been previously determined at 25 °C and 85 °C. The thermo-rheograms presented correspond to mean values of three replicates per formulation (Fig. 3).

All samples exhibited qualitatively the same behavior with a predominance of the elastic component (G′) over the viscous component (G″). At the initial heating stage loss tangent ($\tan \delta = G''/G'$) ranged between 0.31 and 0.33, Samples started to exhibit a more viscous-like behavior due to increased protein mobilization, both formulations showed a common tendency to diminish progressively until a certain temperature was reached (53.5 °C for BNC and 53 °C for the control). This decrease in the moduli values of doughs heated to gelatinization temperatures indicated decreased interactions in the system (Dogan, 2002). In Salvador et al. (2006) own words: “it could be that flour amylase activity on damaged starch at an early baking temperature was freeing absorbed water and reducing the G′ and G″ viscoelastic constants”. Once a threshold had been exceeded, the G′ and G″ values increased until a maximum value was reached (Fig. 3); this stage corresponds to complete starch gelatinization (León, Barrera, Pérez, Ribotta, & Rosell, 2006) and protein denaturing, although the former is cited as being of greater importance (Schofield, Bottomley, Timms, & Booth, 1983). The temperature at which the rise in G′ and G″ takes place has been linked to increased viscosity due to amylose escaping from the granules and forming a gel. Continued heating from 53 °C to nearly 80 °C led to a peak in the viscosity time curve that is related to the interaction of granule swelling and the breakdown of the swollen granules under shear. Around 77-79 °C a second transition was observed.

Continued shearing up to 85 °C led to a decrease in viscosity and both moduli related to granule disruption and thixotropic behavior of the dough (minimum viscosity). On cooling, after the isothermal stage, the sample again increases in viscosity (setback) and the same behavior is observed with G′ and G″ (Fig. 3). At the end of the test (25 °C), the cooked dough with BNC showed the larger moduli (G′ = 258700 Pa y G″ = 44660 Pa) than the Control (G′ = 185000 Pa y G″ = 31200 Pa). BNC reinforced the thermally treated matrix, turning it more rigid, thus the smaller firmness obtained in the TPA must be attributable to the larger pore volume present.

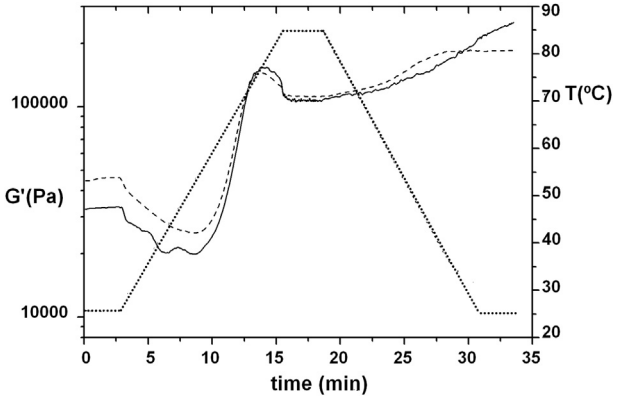

Fig. 3. Thermo-rheograms (G′ vs. time) corresponding to formulations: BNC —— and Control - - - -. Thermal history is also indicated as·····.

3.3. Image analysis by confocal laser microscopy

3.3.1. Dough microstructure

Different techniques can be applied to study the interaction of hydrocolloids with dough components, particularly with gluten proteins, at a microstructural level. The use of different microscopic techniques is a very useful approach to dough microstructure allowing a general overview of the matrix characteristics (Correa, Ferrer, Añón, & Ferrero, 2014). It is usually assumed that upon application of a mechanical action during mixing, hydrated protein aggregates partially dissociate, unfold, and stretch to form a fibrillar and eventually lamellar protein phase through the dough. This protein phase could, at high enough concentrations, constitute the continuous phase of the dough. Dispersed in it is a very large concentration of starch granules. (Amemiya & Menjivar, 1992).

As an example, Fig. 4 shows representative images of dough microstructure obtained with confocal laser scanning microscopy (CLSM) techniques for samples with and without BNC. Both formulations exhibited areas with gluten films and gluten filaments (in red), wrapping starch granules (in green). Control doughs exhibited a highly filamentous and oriented gluten network with a large number of entanglements. Otherwise, the addition of BNC led to a less crosslinked matrix, with thicker and better aligned gluten filaments, with a larger separation between filaments. Control dough looked more compact than the BNC formulation, that is, respect to control BNC samples exhibited a more open matrix. Correa et al. (2014) informed a similar effect regarding the addition of HPMC to wheat bread doughs.

BNC, with its high density of surface OH− could establish electrostatic interactions with gluten proteins also inducing a repulsive effect between contiguous chains. A less crosslinked network than control, with a marked orientation, could be indicating this repulsive effect. It is interesting to remember that the BNC dough presented more elastic characteristics than the control, so the addition of BNC, although apparently weakening the gluten network reinforced the system as a whole.

3.3.2. Crumb microstructure

In Fig. 5 micrographs of both types of crumbs taken by CLSM can be seen. In both micrographs starch (green) occupies the surface of the alveolus and gluten filaments (red) are extended from side to side of the pore. Starch is gelatinized and has lost its crystallinity; its morphology is less recognizable than in the dough (Dürrenberger, Handschin, Conde-Petit, & Escher, 2001). Just a few starch granules remain ungelatinized, imbibed in gluten filaments that cross the pore and are distinguished as discrete green particles. During baking, proteins denaturalize and fix gluten structure, while starch gelatinization increases viscosity and impair dough extensibility (Curic, Novotni, & Smerdel, 2013), as water migrates from gluten to starch. Extensibility loss (gluten fibers are shorter than in the dough) causes rupture of the vapor bubble membranes, gases escape, and the formation of a porous interconected matrix. In the micrographs the empty spaces inside the pores are visualized in gray.

Control crumb showed very thin strands, like small needles,

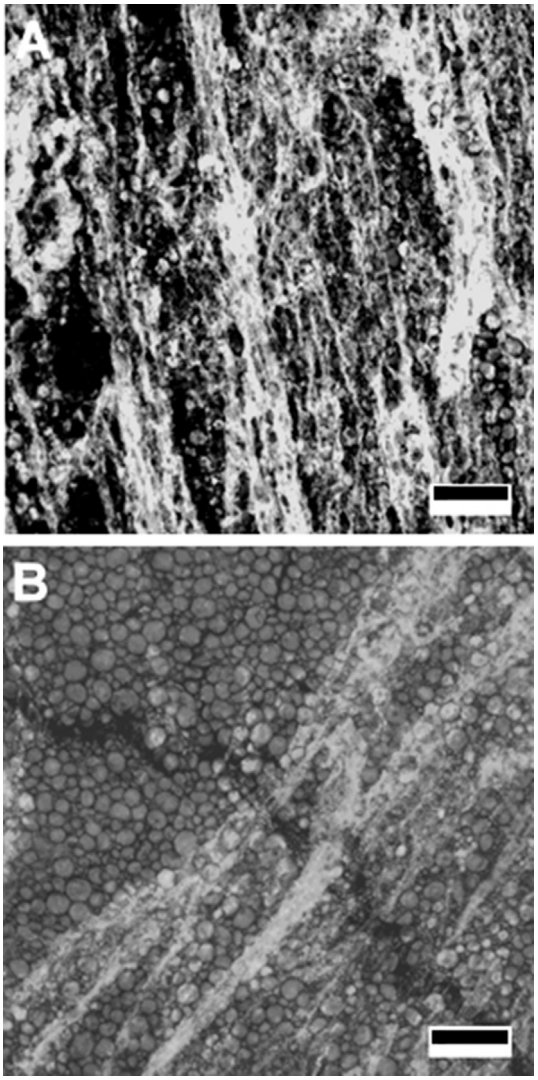

Fig. 4. CLSM of bread dough A) control without BNC and B) with bacterial nanocellulose. Black bar indicates 100 μm

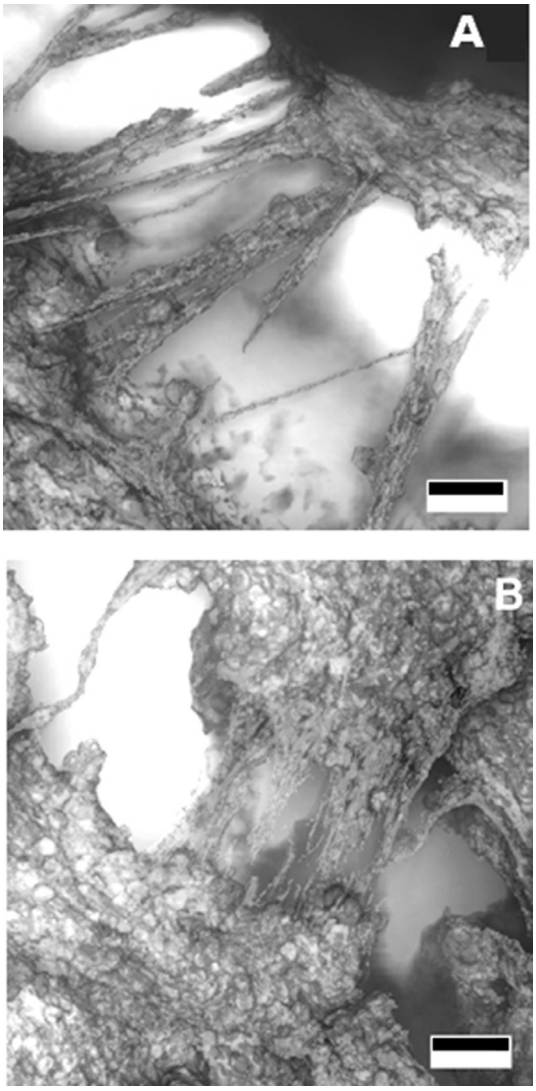

Fig. 5. CLSM micrographs of bread crumb. A) control, and B) with BNC added. Black bar indicates 100 μm.

totally straight (Fig. 5A). In contrast BNC crumb presented interweaved gluten filaments that although oriented, they were thicker and not completely straight. This thickening of gluten filaments could explain the increased gas retention of the matrix during baking, producing a more porous crumb which resulted in a less firm, more tender crumb texture.

4. Conclusion

The higher moisture content found in the crumb with BNC is consistent with the presence of a high hydrophilic nanometer-sized hydrocolloid such as this. Crumb containing BNC presented interweaved thicker gluten filaments than the control formulation without BNC that helped to retain gas, producing a softer and more porous crumb (larger equivalent volume mean diameter D[4,3]), maintaining the same yield as the control. According to the rheological assays, cooked unleavened dough with BNC, after cooling to room temperature, had a more rigid matrix than the control, thus, the less firm crumb measured by TPA must be attributable to the differences in crumb microstructure.

It can be concluded that the incorporation of bacterial nanocellulose produced an improving effect on bread quality, promoting higher specific volume, porosity, luminosity, and moisture retention, and more tender, less firm crumb; characteristics that make it more acceptable to the consumer.

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