A FUNCTIONAL CHITOSAN-ENRICHED FISH SAUSAGE TREATED BY HIGH PRESSURE

López-Caballero*, ME; Gómez-Guillén, MC; Pérez-Mateos, M and Montero, P

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Instituto del Frío (CSIC). José Antonio Novais, 10. 28040 Madrid (Spain). Tel.: +34 91 5445607; Fax: +34 91 5493627.

*Author for correspondence: ifrel54@if.csic.es

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Short title: Chitosan in pressurized fish sausages

Abstract

15 Cod sausages with added chitosan (1.5%), a chitin derived polysaccharide from crabshells, were obtained by high pressure treatment (350 MPa / 7 °C / 15 min). Color, rheological properties, biochemical indices (pH, total volatile basic nitrogen, thiobarbituric acid index) and microbiological counts (total bacteria, lactic acid bacteria, enterobacteria, pseudomonads, staphylococcus) were determined during chilled storage at 2°C. The
20 addition of chitosan in dry form led to a noticeable increase in elasticity and yellowness. When chitosan was added in soluble form, total volatile basic nitrogen remained stable during 25 days of storage. Regarding microbial counts, soluble chitosan did not show any appreciable effect in high pressure sausages. However, when chitosan was added in dry form, higher counts were recorded.

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Keywords: chitosan, preservative, high pressure, sausage, fish, chilled storage.

Introduction

30 High pressure ranging from 150 to 600 MPa can be used to make new products from fish muscle in which color, flavor and nutritional value are only minimally affected (Cheftel and Culioli 1997; Messens and others 1997), and with particular characteristics that differentiated them from products made by conventional thermal treatment. Such new products are largely based on gel-forming capacity of muscle proteins. The use of high
35 pressure to induce gelation of proteins in foods first aroused interest in the 1990s, as an alternative to thermal treatment, the latter causing in principle more severe denaturation (Okamoto and others, 1990; Oshima and others, 1993). The application of high pressure is associated with a small reduction in volume, due mainly to changes in protein hydration and “packing efficiency” of amino acids (Masson 1992). Protein unfolding is much less
40 intense in moderate pressurization than in heating (Heremans and others 1997), and denaturation, unlike thermal denaturation, may be reversible to some extent (Mozhaev and others 1996). At non-denaturing temperatures, the higher the pressure, the larger was the protein unfolding effect (Fernandez-Martín and others 1998).

45 For proper gelation, the minced muscle must be homogenized with salt to allow solubilization of the myofibrillar protein and facilitate subsequent polymerization (Suzuki 1981). NaCl levels in the making of fish gels are normally around 2.5-3 % of the starting weight of the fish mince. High concentrations of salt can have undesirable effects, not only causing changes in texture and promoting oxidative reactions but also causing blood-
50 pressure problems in susceptible individuals (MacFarlane and others 1984). On the other hand, pressure-induced protein denaturation or solubilization could be particularly useful for enhancing the functionality of muscle proteins in order to allow reduction of salt content in restructured products (MacFarlane and others 1984, Cheftel and Culioli 1997).

55 In addition, high pressure technology can be viewed as a means of prolonging the chilled shelf-life of fish products, due essentially to the ability of pressurization to reduce and/or inactivate the microbial load (López-Caballero and others 2000; Hurtado and others 2000) Although the efficiency of high pressure against microorganism is highly dependent on pressure level and temperature, a considerable higher microbial load in pressure-induced
60 fish gels (200 MPa / 3 °C or 375 MPa / 38 °C) than in those induced thermally (90°C 50 min) has been reported (Montero and others 1998), due largely to the high temperature used in heat induction. Nevertheless, the microbial load in a raw product would be considerably higher than in a pressured one.

65 Chitosan is a cationic polysaccharide with a great variety of properties. From a technological point of view, it has been proposed as antimicrobial, texturizer and binder agent (Hardinge-Lyme 2001). Antioxidant capacity of chitosan has been also reported (Kamil and others 2002). From the nutritional point of view, chitosan has been considered as dietary fiber (Deuchi and others 1994; Kanauchi and others 1995), and as
70 hypocholesterolemic agent, by diminishing bile acids in intestine (Shahid solubilized in the food i and others 1999). To be nutritionally active, chitosan needs to be introduced solubilized in the food. Bearing this in mind, it can be introduced just solubilized in the food, or in the form of a powder, which in the stomach become soluble with the acid pH. In these conditions, it is able to “capture” the fat by reacting with triglycerides, cholesterol and
75 bile acids, and afterwards will form an insoluble complex in the intestine, as a consequence of the alkaline pH, acting therefore as a dietary fiber. Therefore, chitosan can be considered a promising ingredient to develop functional foods.

In studies dealing with stability of sausages, chitosan has been used in combination with other substances, such as sodium lactate (Kook and others 2003), carnitine and sulfite (Roller and others 2002), or even technologies as irradiation (Cheorun and others 2001). But to our knowledge, no information is available about the behaviour of chitosan under high pressure treatment.

The objective of the present work was to obtain a new fish product enriched with chitosan, with a sausage-like appearance, by using chilled temperature-high pressure treatment, and to study its properties (color, rheological properties, lipid oxidation and microbial quality) during chilled storage.

Material and methods

Preparation of a blended chitosan-gelatin solution: a chitosan solution and a gelatin solution were prepared separately. A 4 % chitosan solution was made using 95 % deacetylated chitosan from crabshells (Guinama, Valencia, Spain) in 0.5 M acetic acid.

The mixture was held at room temperature for 16 h, stirred for 5 min, and then degassed ultrasonically for 15 min. Separately, dry gelatin was obtained from megrim (*Lepidorhombus boscii*) skins according to Gómez-Guillén and others (2002). The dry fish gelatin (8 %) was dissolved in water, first being allowed to swell at 7 °C for 15 min and then warmed to 55 °C for 30 min. An amount of 25 % glycerol was added to the gelatin solution, and warming at 55 °C continued for a further 30 min. Gelatin was used to thicken the chitosan solution. Additionally, from a sensorial point of view, gelatin improves palatability of the restructured product (Borderías and others 1994).

The blended chitosan–gelatin solution was then prepared with 70 parts of the gelatin/glycerol solution and 30 parts of the chitosan solution. Final pH of this solution was 4.4.

Preparation of the fish sausages: Cod fillets were purchased at a local market, and batches of 4 Kg were cut into small pieces and comminuted at low speed (1500 rpm) at 2 °C in a Stephan mincer (Model UM5; Stephan und Söhne GmbH & Co., Hameln, Germany) for 2 min. Amounts of 3 % NaCl (Panreac, Barcelona, Spain) and 5 % crushed ice were added to the mince, and the mixture was further homogenized at high speed (3000 rpm) for 3 min. Next, 2 % egg white (Sanofi, Barcelona, Spain) and 10 % starch (Clearam CH 20, Laisa, Valencia, Spain) were added and homogenization continued for a further 5 min.

The resulting batter was blended with the rest of the mince (40:60) and divided into three batches, which were stuffed into flexible plastic casing (Krehalon Soplaril, Barcelona, Spain) of 40-µm thickness and 3.5-cm diameter. The first batch was used as control without chitosan (Batch: C). The second batch was added with chitosan powder (1.5 %) (Batch: P), and the third one was added with the chitosan-gelatin blend solution (to achieved 1.5 % of chitosan) (Batch: S). The day the sausages were prepared was considered as day 0. They were kept chilled at 2 °C, and at third day they were pressurized at 350 MPa, 15 min, 7 °C. High pressure treatments were performed in a high pressure pilot unit (ACB 665, Gec Alsthom, Nantes, France) where the temperature (7±3°C) of immersion medium (distilled water) was controlled via a thermocouple with a programmed thermostatization equipment (model IA/2230 AC, INMASA, Barcelona). Pressure was increased by 2.5 MPa/s. After high-pressure treatment, sausages were stored in a cold room at 2±1°C.

Proximate analysis of the raw cod was performed according to the procedures of the Association of Official Analytical Chemists for moisture (method 24003), ash (method 1821), and protein (method 24024) [AOAC 1989]. Crude fat was determined according to the method of Bligh and Dyer (1959). Proximate analysis results were: total protein 16.47 ± 0.25 %; moisture 82.34 ± 0.23 %, total fat 0.82 ± 0.06 %, and ash 1.05 ± 0.01 %.

pH: The pH of the cod was measured using a pH meter (MeterLab pHM 93, Radiometer Analytical, Denmark) using a mixture of 10 g of muscle in 100 mL of distilled water.

Color: The color parameters lightness (L) and yellowness (b) were measured using a Hunter Lab colorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA).

Texture profile analysis (TPA): Pieces of sausages (3 cm diameter x 1.5 cm high) were placed on the flat plate of the texturometer (Instron model 4501 Universal Testing Machine, Instron Co., Canton, MA, USA). Compression was applied using a cylindrical plunger (58 mm diameter) adapted to a 5 kN load-cell at the deformation rate of 50 mm/min. The samples were compressed to 30 % height. The parameters determined were hardness (N), cohesiveness (adimensional) and adhesiveness (g/cm).

Stress-relaxation test: Elasticity was determined by means of a stress-relaxation test after relaxation for 1 min. Percentage relaxation was calculated as $Y_T = 100 \cdot (F_0 - F_1) / F_0$, where F_0 was the force registered at the onset of relaxation immediately after sample compression and F_1 was the force registered after relaxation for 1 min. Thus, $100 - Y_T$ was taken as a percentage index of gel elasticity.

TBA index: Thiobarbituric acid was measured using a modified version of the method of Vyncke (1970) with a glass fibre filter (Type A/E, 1 µm, Pall Corporation, NY, USA) and incubation at 15 °C for 20 hours. Results have been expressed as mg malondialdehyde per Kg of gel.

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Total volatile basic nitrogen: The TVBN determination was based on the method of Antonacopoulos and Vyncke (1989). Portions of 10 g cod sausages were homogenized with 90 mL perchloric acid (6 %) to precipitate the muscle proteins. This was followed by centrifugation at 4,000 x g at 5 °C for 5 min and distillation of the supernatant (Tecator AB, Kjeltec System, model 1002, Höganäs, Sweden). The distillate was collected in boric acid (3 %) and titrated with hydrochloric acid (0.05 N). Results have been expressed in mg of nitrogen per 100 g of sample.

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Microbiological assays: At least four sausages per batch were used for microbiological analysis as follows. An amount of 10 g from each sausage was collected and placed in a sterile plastic bag (Sterilin, Stone, Staffordshire, UK) with 90 mL of buffered 0.1 % peptone water (Oxoid, Basingstoke, UK) in a vertical laminar-flow cabinet (mod. AV 30/70 Telstar, Madrid, Spain). After 1 min in a Stomacher blender (model Colworth 400, Seward, London, UK), appropriate dilutions were prepared for the following microorganism determinations:

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(i) total bacterial counts (TBC) and luminescent bacteria (LM) on spread plates of modified Long & Hammer's medium (L&H) (Van Spreekens 1974) incubated at 15 °C for 5 days; (ii) *Pseudomonas* on spread plates of *Pseudomonas* Agar Base (Oxoid) with added CFC supplement for *Pseudomonas* spp. (Oxoid) incubated at 25 °C for 48 h; (iii) enterobacteria on double-layered plates of Violet Red Bile Glucose agar (VRBG, Oxoid) incubated at 30 °C for 48 h [after first adding 5 mL of Tryptone Soy Agar (Merck, Darmstadt, Germany) and incubating at room temperature for 1 h]; (iv) lactic acid bacteria on double-layered

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plates filled with MRS Agar (Oxoid) incubated at 30 °C for 72 h; (v) coagulase-positive staphylococci on pour plates filled with Baird Parker RPF Agar (bioMérieux, Marcy l'Etoile, France) after incubation at 37 °C for 48 h without subsequent confirmation (ISO 1999). All microbiological counts have been expressed as the log of the colony-forming units per gram (log cfu/g) of sample.

Sampling took place on days 3, 20 and 25 of storage (microbiological and NBVT analyses were performed on day 0 as well). All analyses were performed at least in triplicate.

Statistical analysis

All descriptive statistics and statistical tests were performed using the SPSS computer program (SPSS Statistical Software, Inc., Chicago, Ill.) One-way or two-way analysis of variance was carried out to determine differences. The difference of means between pairs was resolved by means of confidence intervals using Bonferroni test Level of significance was set for $p \leq 0.05$.

Results and discussion

After high-pressure treatment the sausages had the appearance of a highly bound and cohesive gel, though only 40 % of the sausage was comprised of a batter that acted as a binder of the mince.

Table 1 presents the results for lightness (L value) and yellowness (b value) during chilled storage of the high-pressure sausages containing chitosan powder (P) or chitosan dissolved in acetic acid (S). Sausages without added chitosan (C) were also tested as controls. Changes in redness (a value) were not considered on account of the white color

of cod muscle. In order to compare the effect of high pressure on color development, L and b values were also measured in the corresponding unpressurized batters. With high-
210 pressure treatment the L value increased from around 32-34 units in the batters to around 40 units in the pressurized sausage; however the b value underwent a slight decrease, with values ranging approximately 4.5 in the batter without any added chitosan and 5 in the batters with the added chitosan.

215 No significant differences ($p \leq 0.05$) in the lightness values were found between the sausages containing chitosan and the control sausages at any time during the storage period. Conversely, the yellowness value increased with the addition of chitosan, especially when the chitosan was incorporated in soluble form. Still, the alterations during storage were not significant ($p \leq 0.05$). A noticeable rise in the lightness and yellowness
220 values with the addition of chitosan has previously been reported in thermally processed surimi gels (Benjakul and others 2001) and in pork sausages (Jo and others 2001).

Figure 1 plots the texture profile analysis results, i.e., hardness, cohesiveness, and adhesiveness, over the storage period for two of the batches of pressurized sausages, the
225 control batch without any added chitosan (C) and the batch with chitosan added in powder form (P). When removed from the casings, the sausages that contained added chitosan in solution in acetic acid (S) lacked suitable consistency to enable compression tests to be performed. According to the results depicted, adding chitosan powder to the cod sausages did not yield significant differences ($p \leq 0.05$) in hardness, cohesiveness, or adhesiveness
230 compared to the control batch early in storage. However, the sausages in batch P had clearly higher elasticity values than the control batch without added chitosan (Figure 2).

In order to ascertain the effect of chilled high-pressure treatment in producing a sausage-like product from comminuted cod mince, rheological analyses were also performed on the corresponding unpressurized batters (control batter: Cb, and batter containing chitosan powder: Pb) after three days of storage (hardness: Cb 16.73 ± 2.95 N, Pb 22.99 ± 1.17 N; cohesiveness: Cb 0.44 ± 0.01 , Pb 0.50 ± 0.01 ; adhesiveness: Cb 366.33 ± 23.19 g/cm, Pb 493.44 ± 14.59 g/cm; elasticity: Cb 32.06 ± 2.99 %, Pb 39.17 ± 1.48 %). Based on these results, high-pressure treatment chiefly increased cohesiveness and elasticity, and then to a lesser extent hardness and adhesiveness of the corresponding batters. The pressurized sausages had the appearance of a cohesive, elastic fish gel, though only 40 % of the sausage was a salt-containing batter that acted as a binder of the mince. According to the formulation used to prepare the sausages, the salt content of the final product was approximately 1.2%. Therefore, as has been reported earlier, pressure-induced protein denaturation allowed the salt content of the restructured product to be reduced substantially (MacFarlane and others 1984; Cheftel and Culioli 1997). On the other hand, since high pressure affects flavor only minimally (Cheftel and Culioli 1997; Messens and others 1997), this sensory characteristic in pressure-treated sausage resembled largely that of fresh fish. This special feature sets them apart from sausages obtained by conventional heat processing, which may give rise to a typical sulphur aroma as a consequence of extensive disulphide bond formation at high temperature.

Under chilled storage both types of pressurized sausages (batches C and P) exhibited a clear tendency to become harder with increased adhesiveness and elasticity, whereas cohesiveness remained practically unaltered. Changes taking place in pressure-induced gels during storage are generally known to be associated with protein aggregation, which is an essential contributor to changes in textural properties. For instance, increased breaking strength of pressure-induced gels made from Alaska pollock (Shoji and others

1991) and bream (Carlez and others 1995) during chilled storage has been reported previously. There was an approximately two-fold increase in hardness and adhesiveness in the sausages with the added chitosan powder after 20 days of storage. An increase in the breaking strength of surimi gels containing chitosan has also been observed, suggesting that enhancement of gel formation by chitosan could be related to endogenous transglutaminase (TGase) activity (Kataoka and others 1998). These researchers postulated that the gel-strengthening effect could be associated with the presence of the reactive amino group in the C-2 position on the glucosamine unit (as an acyl acceptor) of the chitosan, which may react with the glutamyl residues of myofibrillar proteins to form protein-polysaccharide conjugates. However, initial differences in hardness between the sausages in batches C and P in the first three days of storage were not significant ($p \leq 0.05$). One possible explanation is the type of chitosan used, having a high degree of deacetylation (around 95 %). Smaller increases in the breaking strength of low temperature setting suwari and kamaboko (cooked) gels containing chitosan with increasing deacetylation have been reported (Benjakul and others 2001). This has been related to increased ability by chitosan amino groups to form TGase mediated cross-links with the glutamyl residues of the myofibrils, resulting in less inter- or intramolecular polymerization of the myofibrils themselves. Based on all these reported results, the marked difference between the sausages in batches C and P after 20 days of chilled storage could have been produced by enhanced endogenous TGase activity in the presence of chitosan which was able to proceed at the chilled temperature over the lengthy storage period. Furthermore, as far as the high-pressure treatment is concerned, TGase activity has been shown to be hindered hardly at all by pressures of around 300 MPa and to remain active enough to continue later during setting (Gilleland and others 1997; Ashie and Lanier 1999).

At the end of the storage period, hardness and adhesiveness had decreased appreciably in the batch P sausages, but the differences with respect to the control sample were not significant ($p \leq 0.05$). Montero and others (1998) also reported a distinctly discernible decrease in hardness in blue whiting gels induced by high pressure after chilled storage for 20 days. These textural changes were attributed to cleavage of strong bonds as a result of microbial action (total viable microorganism counts of around 6 log cfu/g), resulting in protein degradation.

The pH in batches C and P held practically constant over the entire storage period at around the initial level close to neutral. In contrast, in batch S the pH decreased to around 5.5 because of the solvent used to dissolve the chitosan (acetic acid), and remained practically unaffected along the storage period. The changes in pH in the pressurized sausages were unrelated to the accumulation of volatile bases with microbial growth.

The initial volatile basic nitrogen content was about 11 mg TVBN/100 g fish. During storage the formation of volatile bases was observed to increase in the control batch (C) to over 30 mg / 100 g muscle after around five days of storage. In the chitosan-containing sausages, batch P followed the same trend as the control batch, whereas the change in the TVBN content in batch S was negligible for 25 days (Figure 3). Jeon and others (2002) reported a 33-50 % reduction in TVBN formation in cod fillets coated with different forms of chitosan having viscosity molecular weights ranging from 6.6×10^5 to 1.8×10^6 Da.

Oxidation values in the high pressure-treated sausages increased compared with the minced muscle (0.01 mg malondialdehyde/kg) [Figure 4]. Pressure has been found to induce oxidation of lipids in fish gels (Cheftel and Culioli 1997; Pérez-Mateos and others 2002).

The batch made with the chitosan dissolved in acetic acid exhibited higher values ($p \leq 0.05$) than the other batches throughout storage. The explanation for this is that the acetic acid can also react with TBA and also that the acid medium could release higher amounts of TBA-reactive substances, or TBARS, bound to other food ingredients (Guillén-Sans and Guzmán-Chozas 1998).

Total bacterial counts (TBC) in the cod fillets were 4.2 log cfu/g, lower than reported by other studies in this raw material (Debevere and Boskou 1996). Counts of *Pseudomonas* spp. and lactic acid bacteria were 3.5 and 2.4 log cfu/g, respectively. Enterobacteria and staphylococci were both below the detection threshold for the method employed (<1 log cfu/g).

Table 2 presents the counts in the sausages before (day 0) and after high-pressure treatment over the 25-day storage period. Sausage preparation led to higher counts than in the raw material (especially in the case of the TBC and pseudomonads), but the high-pressure treatment (350 MPa at 7 °C for 15 min) caused the numbers of microorganisms to fall again. Various workers have reported microbial inactivation following high-pressure treatment in seafood (López-Caballero and others 2000) and in minced meat (Carlez and others 1994; López-Caballero and others 2002).

The behaviour of batches C and S was similar, with a decrease of more than 3 log cycles in the counts. Chitosan has been described as having antimicrobial properties (Jeong and others 2002; Coma and others 2003). Previous results obtained have shown that applying an edible coating of chitosan to fish patties brought about a drop in the microflora, especially in the Gram-negative bacteria (unpublished data). In relation to this effect,

Helander and others (2001) reported that chitosan sensitized bacteria to the action of certain chemical compounds (i.e., anionic detergents, dyes, etc.). However, in this study no extra effect attributable to the dissolved chitosan over and above that of the high-pressure treatment was observed. Indeed, when chitosan was added to the mixture in powder form, the decrease in the TBC was only about 2 log cycles (Table 2).

The cells that remained intact multiplied or the cells that were damaged by pressurization recovered during storage (total bacteria counts). The trends for batches C and S were similar, with TBC levels that did not exceed the initial levels after 20 days of storage. The findings of Carlez and others (1994) confirmed the recovery of microorganisms subsequent to the repair of microbial cells, with the lag time for repair being related to the intensity of the high-pressure treatment and other factors. The recovery and/or multiplication of microorganisms was more clearly discernible in batch P, in which lactic acid bacteria were recorded in addition to the total bacteria. The slightly acidic conditions produced by the acid medium in which the chitosan had been dissolved was not particularly conducive to growth of the lactic acid flora in batch S. At the same time, however, the antimicrobial effect is not ascribable to the solvent, i.e., to the acetic acid at pH 5 (Coma and others 2003). The slightly higher counts in batch P as compared to the control batch were not reflected in greater accumulation of volatile bases (Figure 3).

High-pressure treatment is capable of destroying microorganisms (Cheftel 1995). The Gram-positive flora is more resistant to pressure than is the Gram-negative flora (Carlez and others 1993; López-Caballero and others 2002). In addition, chitosan is thought to exert an antimicrobial effect by binding to the surface of the cytoplasmic membrane (Coma and others 2003). While these investigators reported that the outer membrane protects Gram-negative cells, the ability of chitosan to disrupt the permeability barrier of the outer

membrane in Gram-negative bacteria has also been described (Helander and others 2001). Therefore, the use of added chitosan in conjunction with high-pressure treatment could combine to bring about a pronounced decline in the levels of active Gram-negative
365 spoilage in fish and in the resistant Gram-positive flora (e.g., psychrotrophic pathogens like *Listeria monocitogenes*, etc.), thereby enhancing the stability and safety of these new products. The lesser effectiveness of the high-pressure treatment in batch P, which had higher counts than the control batch (C) itself after 25 days, is suggestive of a certain
370 protective effect of the chitosan (marine fiber) matrix on the microorganisms. This effect has also been observed during storage of cod patties that did not undergo high-pressure processing (unpublished data). A related aspect is that the bactericidal effect of chitosan is negligible at neutral pH levels because of chitosan's poor solubility and the presence of a sizeable proportion of uncharged amino groups (Sudarshan and others 1992; Helander
375 and others 2001).

The chilled temperature high-pressure treatment thus was able to produce a chitosan-enriched sausage. This fish product can be considered functionally active nutritionally, irrespective of whether the chitosan is added in soluble form or as a powder to be
380 dissolved in the stomach. The product has an extended shelf-life thanks to the high-pressure treatment. Chitosan powder was observed to reduce the inhibitory effect of the high-pressure treatment on microbial growth, which we have attributed to possible action by the polymer that serves to protect the microorganisms from the effects of the high-pressure treatment. This possibility will be examined in subsequent experiments.

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Table 1.- Color of high pressure sausages (350 MPa, 15 min, 7 °C) containing 1.5% chitosan, stored at chilled temperatures (2 ± 1 °C).

C: control without chitosan, S: solubilized chitosan, P: chitosan powder

	Lightness (L)			Yellowness (b)		
	Days of storage					
	3	20	25	3	20	25
C	41.04 a/xy	41.87a/x	40.17a/x	2.97a/x	2.34a/x	2.54a/x
S	42.33 a/y	40.59a/x	40.89a/x	5.13a/z	4.62a/z	4.75a/z
P	39.58 a/x	40.70a/x	39.39a/x	3.93a/y	3.57a/y	3.71a/y

Different letters a,b... in the same row indicate significant differences ($p \leq 0.05$) on each lot as a function of storage time; different letters x, y, z... in the same column indicate significant differences ($p \leq 0.05$) among lots on each sampling date.

575 **Table 2.** Microorganisms (log cfu/g) in high pressure sausages (350 MPa, 15 min, 7 °C) containing 1.5% chitosan stored at chilled temperatures (2 ± 1 °C).

C: control without chitosan; S: solubilized chitosan; P: chitosan powder.

Batches	Microorganisms	Days of storage			
		0	3	20	25
C	Total bacteria count	5.4±0.1	<2	3.61±0.0	4.20±0.1
	Lactic acid bacteria ⁽¹⁾	2.1±0.0	<1	<1.5	1.53±0.1
	Enterobacteria ⁽²⁾	2.1±0.0	<1	<1.5	<1
	Pseudomonads ⁽³⁾	4.3±0.0	<2	<2.5	<2.5
	Staphylococcus ⁽⁴⁾	<1	<1	<1	<1
S	Total bacteria count	5.4±0.1	<2	3.02±0.3	4.02±0.0
	Lactic acid bacteria	2.1±0.0	<1	<1	<1
	Enterobacteria	2.1±0.5	<1	<1	<1
	Pseudomonads	4.3±0.0	<2	<2	<2.5
	Staphylococcus	<1	<1	<1	<1
P	Total bacteria count	5.4±0.1	3.02±0.1	5.39±0.1	5.98±0.3
	Lactic acid bacteria	2.1±0.0	<1	2.43±0.2	3.50±0.5
	Enterobacteria	2.1±0.0	<1	<1	<1
	Pseudomonads	4.3±0.0	<2	<2	<2.5
	Staphylococcus	<1	<1	<1	<1

(1) MRS Agar

(2) Violet Red Bile Glucose Agar (VRBGA)

580 (3) Pseudomonas Agar Base

(4) Baird Parker RPF Agar

Figure 1.- Hardness (N), cohesiveness (adimensional) and adhesiveness (g/cm),
determined by texture profile analysis in an Instron texturometer (model 4501), of high
pressure sausages (350 MPa, 15 min, 7 °C) stored at chilled temperatures (2 ± 1 °C).
C: control without chitosan, P: with 1.5% chitosan powder. Different letters (a, b, c) indicate
significant differences ($p \leq 0.05$).

595 Figure. 2.- Elasticity (%), determined by stress-relaxation test in an Instron texturometer (model 4501), of high pressure sausages (350 MPa, 15 min, 7 °C) stored at chilled temperatures (2 ± 1 °C).

C: control without chitosan, P: with 1.5% chitosan powder.

Different letters (a, b, c) indicate significant differences ($p \leq 0.05$).

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Figure 3. Total volatile bases (mg TVB-N/100 g) of high pressure sausages (350 MPa, 15 min, 7 °C) containing 1.5% chitosan stored at chilled temperatures (2 ± 1 °C).

C: control without chitosan, S: solubilized chitosan, P: chitosan powder.

Different letters a, b, c.. indicate significant differences ($p \leq 0.05$) on each lot as a function of storage time; different letters x, y, z... indicate significant differences ($p \leq 0.05$) among lots on each sampling date.

Figure 4.- Evolution of thiobarbituric acid index (mg malonaldehyde/ Kg sample) of high pressure sausages (350 MPa, 15 min, 7 °C) containing 1.5% chitosan stored at chilled temperatures (2 ± 1 °C).

C: control without chitosan, S: solubilized chitosan, P: chitosan powder.

Different letters a, b, c.. indicate significant differences ($p \leq 0.05$) on each lot as a function of storage time; different letters x, y, z... indicate significant differences ($p \leq 0.05$) among lots on each sampling date.