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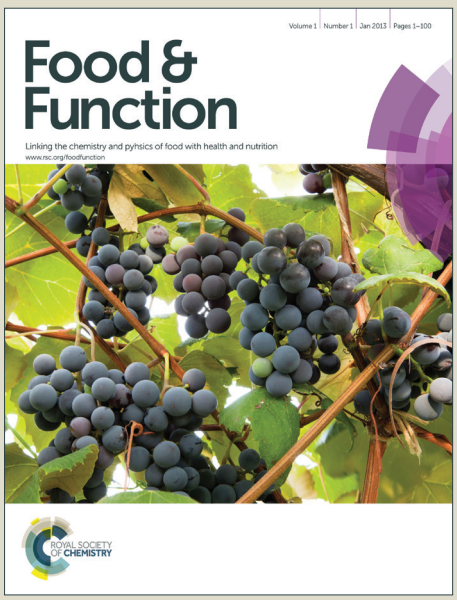

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DEVELOPMENT OF A LOW FAT FRESH PORK SAUSAGE BASED ON CHITOSAN WITH HEALTH CLAIMS: IMPACT ON THE QUALITY, FUNCTIONALITY AND SHELF-LIFE

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A low fat fresh pork sausage based on chitosan was developed with the objective of obtaining a new functional meat product with improved properties and health claims promoting cholesterol reduction. Sausages were formulated with chitosan (2%, w/w) and different fat levels (5%, 12.5% and 20%, w/w). The results indicated that incorporation of 2% chitosan to produced pork sausages with health claims of reduction of cholesterol is technologically feasible. Additionally, the chitosan reduced the microbial growth, revealing interesting fat and water absorption capacities, reduced lipid oxidation, provided greater stability in terms of colorimetric parameters and promoted positive firmer texture and gumminess. Reduction of fat content to levels of 5% was positively achieved with the incorporation of chitosan. Sensorial analysis showed as panelist did not detect any significant difference in taste and any unfavorable effect on the sausages appearance as consequence of chitosan addition and variation of fat.

Keywords: chitosan, functional meat product, fat reduction, pork meat, quality, shelf life

Introduction

Processed meat products are widely consumed foodstuffs relatively inexpensive compared to traditional fresh meat cuts[1]. Fresh pork sausages are a meat product, consisting mainly of pork and a variable amount of fat, which are chopped and mixed with water and/or ice and complemented with a variety of non-meat ingredients.[2] After homogenization, the meat mixture is stuffed into casings and ready-made products are maintained under refrigerated storage condition until consumption.[3] Due to the high fat content, the perishable nature of the raw materials and the lack of thermal processing, such products are

prone to spoilage by both lipid oxidation and microbial contamination.[4] Therefore, several synthetic food additives, such as nitrites, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ), have been used to prevent these harmful events and increase the shelf life of the product.[1,4] However, nowadays, society is becoming aware of the importance of diet for health. This fact, joined to that safety of some synthetic additives, has been questioned in the last few years[1] and have caused an increasing demand of natural products by the consumers as an alternative to chemical preservatives in foods. Among all the possible additives, chitosan, as a biopolymer with interesting high antimicrobial capacity, has attracted the attention of the food industry as an alternative to replace the synthetic additives, in order to meet the needs and standards of food safety.[4-8]

Additionally to its antimicrobial capacity, chitosan possess other interesting properties such as antioxidant capacity[4,9] lipid and water binding capacity[10-12] and emulsification properties.[13] Due to these properties, chitosan have been described as an interesting functional and technological ingredient, since it could act not only as an additive, but could also provide improved properties and a better nutritional profile to the final product.[14] Regarding the nutritional and functional benefits, numerous research *in vitro* studies have reported the ability of chitosan to decrease the serum cholesterol.[15-17] *In vivo* studies in animals have reported that chitosan exhibits hypocholesterolemic and hypolipidemic effect, including the reduction of blood and liver triglycerides (TG) and total cholesterol (TC) levels in animals.[18-20] Other studies have also reported the hypocholesterolemic effect of chitosan on humans.[21,22] Recently, due to the consistent evidences on the chitosan capacity to decrease serum cholesterol, the European Food Safety Authority (EFSA) has approved a health claim which establishes that "regular consumption of chitosan contributes to the maintenance of normal blood cholesterol concentrations". In order to

bear the claim, EFSA demands a quantity in food of at least 3 g/day of chitosan in one or more servings.[23] This implies that the functional food is consumed as a part of a balanced diet and on a regular base, and the selected functional ingredient is integrated in a food matrix with an equilibrated nutritional profile allowing that one or more serving doses assure the amount required to provide the health claim.

In the context of a healthy diet-related the demand for low-fat meat products has also increased.[24] Lin and Chao[25] indicated that chitosan could be used positively into a reduced fat Chinese-style sausage. Their results showed better or similar quality of chitosan sausages regarding physicochemical, microbial and sensory characteristics with no adverse effects in textural properties.

To date, some studies can be found on the fat reduction in meat products by the incorporation of chitosan,[9,25] however, to our knowledge, information on the application of chitosan in meat products is not enough to establish if the product accomplish the specifications regulated by the EFSA to be claimed as functional ingredient contributing to the maintenance of normal blood cholesterol concentrations. Furthermore, most of these works did not reflect this potentiality.

For that, in this study, chitosan was included in an adequate concentration (2%, w/w) to accomplish the EFSA claims (3g of chitosan/day in one or more servings) on its hypocholesterolemic effects in a low-fat meat matrix with the main objective of establishing if this inclusion could be technologically feasible and could affect the quality and safety of the product. The chosen meat matrix was pork sausages. Samples were produced with different percentages of fat with required amount of chitosan and stored at 4 ºC during 15 days. Microbiological, physico-chemical and sensorial aspects were analyzed during the entire shelf-life.

Results and discussion

Microbiological analysis

In the sausages developed in this study, the only added preserving compounds were natural salt, spices and chitosan, with no addition of nitrites or sulphides. Table 1 shows the results obtained for the microbiological counts of the different microbial groups assessed on fresh samples after production and throughout storage time. In general, it is possible to observe as microbiological counts increased, in all cases, throughout storage time. Additionally, chitosan incorporation induced, in general, a significant reduction of viable cells (ca. 0.5 – 1.0 Log CFU/mL) in the fresh sausage samples, maintaining these differences throughout the storage time. The group of mesophilic bacteria showed at time 0, values ranged from 7.11 ± 0.03 to 8.25 ± 0.03 log CFU/g for sausages without chitosan and from 6.80 ± 0.09 to 7.82 ± 0.10 log CFCU/g for sausages containing chitosan. The presence of high levels of initial mesophilic bacteria is explained by the natural contamination in raw meat, which is dependent on type of animal, the slicing method and storage time under refrigeration until use, that in the present work for pork it presents high microbial counts. Similar mesophilic count values were reported by Sayas-Barbera *et al.*,[12] which found values ca. 7.0 log CFU/g after 8 days of storage in pork model burgers added of chitosan. However, these values increased slightly and gradually, with time, being the values always significantly lower in all samples containing chitosan. Values between 8.25 ± 0.02 and 8.79 ± 0.07 in sausage controls and between 7.38± 0.03 and 8.00 ± 0.04 log CFU/g in sausages with chitosan were found after 15 days of storage at 4 °C.

With regard to the variation of fat ($p < 0.05$), sausages containing chitosan and less fat content (1B) decreased by 0.5 log units at times 0, 5 and 10 days of storage, being this reduction about 0.8 log units after 15 days. In samples 2B and 3B, the reduction was ca. 1 log CFU/g on days 5, 10 and 15 in relation to the control sample. The antimicrobial effect

of chitosan observed in this study is in accordance with Sayas-Barbera *et al.*,[12] which indicated a reduction of 2 logs in pork model burgers added 1% low molecular weight chitosan at the end of storage (8 days). In fresh pork sausages, Roller *et al.*,[36] reported a 2 log units reduction after addition of 0.6% (w/w) chitosan in combination with sulfites after 24 days of storage at 4 °C. Georgantelis *et al.*[4] reported a decrease between 1 and 2 log units in fresh pork sausage added of 1% (w/w) chitosan after 20 days of storage at 4 ºC. Soultos et al.[7] also indicated that addition of 1% (w/v) chitosan decreased by at least 1 log unit in fresh pork sausages stored for 28 days at 4 °C.

The initial values of psychrophilic bacteria were ranged from 4.81 ± 0.06 to 5.35 ± 0.02 log CFU/g. These values were increasing along the time until reaching maximum values of 8.44 ± 0.01 log CFU/g after 15 days of storage for the samples without chitosan and values of 7.95 ± 0.04 log CFU/g for the samples containing chitosan. Regarding the percentage of fat, plausibly the bacterial counts decreased with the increase in fat content and were, also, lower for the samples containing chitosan. Maximal effect of chitosan was produced at 5 days of storage. Data collected in the literature confirm the behavior obtained. Thus, Soultos *et al.*[7] reported values of pseudomonas (one of the most representative psychrophilic bacteria in food) between 4.07 ± 0.49 and 3.14 ± 0.62 log CFU/g for fresh sausages without chitosan and containing 1% (w/w) of chitosan, respectively. These values increased after 15 days of storage at 4 ºC, reaching values of 5.68 ± 0.82 and 4.67 ± 0.67 log CFU/g, respectively. This increase corresponds to 1.5 log CFU/g in both samples. After 28 days, the cell counts found were 7.56 ± 0.66 and 6.67 ± 056 log CFU/g, corresponding, in this case, to an increase of 3.5 log units for both samples. In another study with traditional Greek fresh sausages, Georgantelis *et al.*[4] found initial values of pseudomonas of about 6.71 ± 0.38 and 5.95 ± 0.30 log CFU/g for the samples without and with 1% (w/w) of chitosan, respectively. After 15 days of storage at 4 ºC these counts

increased until 7.30 ± 0.20 and 6.16 ± 0.36 log CFU/g, being this increase much lower than in the case reported before. However, it is noticeable to indicate that initial counts were also higher.

The group of *Enterobacteriaceae* showed relatively low counts assuring low contamination of meat, and counts were always lower in samples added of chitosan than in control sausages (without chitosan addition) ($p < 0.05$). Regarding to the fat content, the samples containing higher fat amount, showed lower microbial counts, but did not differ significantly ($p > 0.05$), however the counts increased significantly throughout storage time for all samples ($p < 0.05$). García *et al.*[37] also corroborated these results in a study on the evaluation of the effect of partial replacement of sodium nitrite in pork sausages by chitosan. These authors reported initial values of *Enterobacteriaceae* ca. 1 log CFU/g and values greater than 7 log CFU/g after 35 days of storage at 4 ºC. Georgantelis *et al.*[4], in a work about the effect on the addition of rosemary extract, chitosan and α- tocopherol in fresh pork sausages, found values of 5.01 ± 0.23 and 3.80 ± 0.21 log CFU/g in the control sample and in that one containing 1% (w/w) of chitosan, respectively. These values increased during storage at 4 ºC reaching values ca. 5.38 ± 0.22 and 3.90 ± 0.17 after 20 days, respectively. Soultos *et al.*[7] in fresh pork sausages reported initial values between 3.51 ± 0.24 and 2.64 ± 0.11 Log CFU/g for control samples and samples with chitosan, respectively. These values also increased until 4.90 ± 0.36 and 3.94 ± 0.22 log CFU/g after 15 days of storage at 4 ºC.

Finally, regarding the counts of yeast and molds, the values obtained for fresh pork sausages at time zero were between 4.85 ± 0.12 and 5.27 ± 0.03 log CFU/g. During the storage time, these values increased to 6.80 ± 0.07 and 7.35 ± 0.03 log CFU/g after 15 days, being this increase always lower in all sausages containing chitosan than in control samples without chitosan. Similar tendency was reported by Garcia *et al.*[37] in an studies

using chitosan for partial substitution of nitrites in pork sausages, who showed an increase of up to 3 log units in the yeast content, while for molds the value was kept constant. Georgantelis *et al.*[4] found initial values of 4.90 ± 0.04 log CFU/g which increased until 7.93 ± 0.19 after 15 days of storage at 4 °C in samples without chitosan and 6.56 ± 0.08 log CFU/g for samples containing 1 % (w/v) of chitosan. García Fontán *et al.*[38] in samples of "androlla" reported an initial average value for molds and yeasts of ca. 4.30 ± 1.73 log CFU/g (ranging from 1.60 to 6.99). These values also corroborate the initial values found in our study.

Physico-chemical analysis

Proximate composition and pH. The formulation of pork sausage was consisted of 2% (w/w) chitosan, being this percentage necessary to meet the requirements of EFSA (3 g/day). Thus, one serving of 3 sausages (150 g containing 3 g of chitosan) a day could contribute to reduce blood cholesterol level. However, this amount could influence several properties of the sausages, so that the proximate composition analysis was performed.

Chemical composition of fresh pork sausages prepared with different fat levels, with and without chitosan, is shown in Table 2. Moisture levels were similar to those reported for fresh pork meat, as the formulation of the meat product was just pork lean.[39,40] Values obtained for moisture content were different depending on the percentage of fat and the addition or not of chitosan. Thus, regarding the fat content, it is possible to observe as these values were higher for the samples with lower amount the fat. With respect to chitosan, its addition showed to produce a significant (p <0.05) decrease in the moisture content when compared with the corresponding samples used as a control (no chitosan addition and same level of fat). This fact is due to the chitosan ability to absorb water. Sormoli *et al*,.[41] evaluated the effect of chitosan hydrogen bonding on lactose crystallinity during spray drying and reported that chitosan can easily absorb moisture by hydrogen

bonding with water molecules through its hydroxyl and amine groups.[42] There is a greater difference between sample 2A and 2B (12.5% (w/w) of fat, with and without chitosan, respectively). A similar behavior was reported for Sayas-Barberá *et al.,*[12] who, in pork model burgers without fat addition, found similar values to those reported in this work, in samples with less addition of fat. Likewise, they found that addition of chitosan caused a decrease in the moisture of samples. As it can be seen, during storage at 4 ºC the moisture content in fresh pork sausages decreased along the time of storage, probably due to the loss of water during storage according to Andrés *et al.*[32]. Soltos *et al.*[7] reported a similar effect in Greek style fresh pork sausages, where they found moisture values ranging 57.4 - 58.1% (w/w) on day 0 and 54.2–54.7% after 28 days of storage at 4 °C.

Regarding the results obtained for the protein content, the addition of chitosan was no statistically different ($p < 0.05$). The slightly higher values were obtained in sausages with lower fat content, however, these differences also were not significant ($p < 0.05$). Protein levels slightly decreased as a function of the storage time in all samples, however not significant differences were not found ($p < 0.05$). Some authors indicated a possible correlation between protein and lipid oxidation thus reducing the protein content throughout the storage period in pork liver pâté and hamburger.[43-45]

Analysis of samples showed a lower fat content in samples with chitosan compared with their respective controls without chitosan, being statistically different ($p < 0.05$). This effect is due to the ability of chitosan to binding fat.[46,47] During storage the fat content increased, which can be explained by the decrease of moisture. This effect was also observed for the ash content although in this case the increase was slight and no statistically difference ($p > 0.05$).

The differences in fat (5, 12.5 and 20% (w/w)) allow proving that it is possible to produce low fat sausages (5%) with 2% chitosan, assuring a food matrix with an equilibrated

nutritional profile (low fat content) allowing that one serving doses (3 sausages of 50 g) assure the amount required to provide the health claim.

For pH, samples containing chitosan showed higher values (ranging from 6.68 ± 0.04 to 6.52 ± 0.12) than control samples (5.96 ± 0.02 - 5.86 ± 0.07) (data not shown). Similar behavior was reported by others researchers (Jo et al. (2001).[4,7,12] The increase of pH is due to the basic nature of chitosan[12] promoted by the amino groups present, Sayas-Barbera *et al.*[12] established that the increase in pH values is dose-dependent (6.13 ± 0.06 at 1% (w/w) of chitosan) supporting, thus, our results. The higher values obtained in this study can be a consequence of the major chitosan concentration (2% (w/w)). A significant increase ($p < 0.05$) in values was also observed during the storage period for all samples. In this case, the observed effect can be possibly attributed to microbial proteolysis, which causes protein and amino acid degradation resulting in the accumulation of basic compounds such as ammonia.[48]

Lipid oxidation. Lipid oxidation is one of the most relevant reaction in the food chemistry.[49] The unsaturated fatty acids, especially polyunsaturated ones (PUFA) are highly susceptible to the oxidation,[50] reacting with molecular oxygen via a free radical chain mechanism.[51] It contributes to the development of unacceptable organoleptic characteristics and it may also affect the nutritional value or even give rise to toxic compounds in meat and meat products.[52] Therefore, inclusion of ingredients in the meat formulation could have a significant contribution towards the extension of shelf life.[4] Sodium nitrite has been widely used for its antioxidant action.[53] However, the utilization of nitrite has been limited by this result in the formation of *N*-nitrosamines, a group of compounds that are well known for their carcinogenic and mutagenic activities.[54] In this respect, chitosan plays an important role since it has been considered as a potential natural antioxidant[4,9,55] without side effects. In our study, lipid oxidation increased proportionally

with the increase of fat, being much more intense in the control samples than in the samples with chitosan (Figure 1). The most significant values ($p < 0.05$) were observed in samples with 20% (w/w) of fat (3A and 3B), where presence of chitosan (sample 3B) allowed to decrease lipid oxidation in a 55% at 0 days and a 64 % after 15 days of storage at 4 ºC, when compared with control sample (3A). This inhibitory effect is explained by the ability of chitosan to chelate iron ions.[6] The efficiency of this polymer to control lipid oxidation in meat and meat products has been previously reported by several authors.[4-7] Oliveira *et al.*[1] reported that the use of natural additives has attracted especial attention for presenting antioxidant effects similar to or better than those of synthetic preservatives. In our study, the chitosan allowed that the sample containing 20% (w/w) fat (3B) did not present statistically significant differences ($p > 0.05$) over the 15 days of storage. This behavior was also observed in samples with the lowest fat content (5%), 1A and 1B. Additionally, the capacity to reduce efficiently the fat content to values ca. 5% with addition of 2% chitosan, assured a reduced lipid oxidation throughout storage time, with no significant difference from control, due to the low fat content. Thus, the addition of 2% (w/w) chitosan or reduction fat can favor the production of a sausage with better quality and longer shelf life concerning lipid oxidation profile.

Color measurement. The addition of chitosan significantly affected the color parameters ($p < 0.05$) of fresh pork sausage. In the first days of storage the L* values were higher for samples with chitosan (Figure 2a). Kachanechai *et al.*[56] reported that LMWC can better penetrate the meat matrix due to the smaller size of their granules than HMWC resulting in higher values of L*, as it was observed with our samples. During storage, the L* value increased in control samples (without chitosan) while in samples containing chitosan, this parameter, decrease. This difference was more pronounced after 15 days of storage. This increase in the samples without chitosan may be due to the oxidation and concentration of

metamyoglobin in the meat. Sayas-Barbera *et al.*[12] found similar results during the storage of fresh pork burgers and justify their results in the same way. Changes in L* can be also related to surface water, water vapor exchanges between the products and the environment and modifications of the different states of the hemepigments.[57]

Fat content also affected the parameter L* ($p < 0.05$), since samples with higher fat content showed higher values of L*, being the highest brightness values found in samples with 20% of fat. Guerra *et al.*[58] reported similar behavior in goat mortadella prepared with different levels of fat and goat meat from discarded animals, indicating that a high addition of fat provides a great clarity to the sample.

Regarding the other determined color parameters, in Figure 2b it is possible to observe as, during the storage period at 4 ºC, the values of redness (a*) decreased for all samples, however reduction in sausages containing chitosan was lower than in control samples without chitosan. These differences were statistically significant ($p < 0.05$). This effect may be due to the antioxidant potential of chitosan.[9,54,59]. Youn *et al.*[60] reported that addition of chitosan in meat sausage had a more reddish surface than sausages without chitosan. Lee *et al.*[61] investigated the stability of pork meat impregnated with chitosan solutions (30 and 120 kDa) and concluded that the color of the meat kept its value a* without changes during storage. The mechanism related to the preservation of the red color can be explained due to the chelating properties of chitosan. According to Georgantelis *et al.*,[4] chitosan could be chelating iron ions of meat hemoproteins during heat processing or storage. Similar behavior was reported by Sayas-Barbera *et al.*[12] that evaluated the effect of concentration and molecular weight in pork model burgers. Regarding the possible influence of fat on the parameter a*, no statistically significant effect ($p > 0.05$) among samples of the same group, however, the highest values of a* were found in samples with 5% (w/w) of fat (1A and 1B). Given these results, we highlight the absence of chemical

additives in our study and confirmed that chitosan is a natural antioxidant that has a similar action to nitrite in the preservation of the color red. Song *et al.*[61] and Soltos *et al.*[7] reported that the nitrate/nitrite has a long history of use as a precursor in the formation of pink color of cured meats developed for reactions until the formation of the nitrosomyoglobin (NO-Mb) pigment.

The yellow color (b*), was always lower in samples with chitosan than in control samples (Figure 2c). During storage, b* values increased for all samples ($p < 0.05$) indicating that the yellowing of the samples could be related to the intensity of the oxidation process. Fernández-López *et al.*[57] and García-Esteban *et al.*,[63] in a work on the effect of storage time on color properties of pork meat and ham, observe a same trend, and indicated that oxidation could increase b* values by rancidity. Lin & Chao[25] and Sayas-Barberá *et al.*[12] also reported an increasing in b* values with the time of storage.

Water retention capacity. The water retention capacity (Table 2) of cooking pork sausages was affected by addition of the chitosan, by storage period and variation of fat ($p < 0.05$). Values for control samples (78.55 - 97.84%) were always lower, at day 0, than values obtained for samples containing chitosan (95.87 - 120.01 %). The same can be observed after 15 days of storage, being 78.59-112.96 and 96.96 - 126.34 the values found for the control samples and the samples with chitosan, respectively. Sayas-Barbera *et al.*[12] reported similar behavior and indicated that the highest cooking yield for the hamburger containing 1% (w/w) chitosan (61.90 ± 0.18) than the control samples (58.79 ± 1.30) can be justified by the ability of chitosan to retain water. Thus, the property of water retention of chitosan may be dose dependent, explaining this fact that the values in our study are higher than the values presented by Sayas-Barbera *et al.*[12] since we've used 2% instead of 1%. The ability to hold moisture and other juices before and after heat treatment is one important attribute in sausage and other meat products.[64] After 15 days, WRC increases for

both samples control (1, 2 and 3A) and sausages added chitosan (1, 2 and 3B). Ayadi *et al.*[65] reported similar behavior in turkey meat sausages added carrageenan and indicated that this increase is probably due to the water loss during storage. Regarding the fat content, the water retention capacity was higher for the samples with higher amount of fat (3A and 3B). This behavior is consistent with Cavestany *et al.*,[66] which reported that the higher the percentage of fat is the more concentrated and dense will be the emulsion's continuous phase, favoring, thus, the formation of the structure with greater water-holding ability.

Texture profile analysis. Results obtained from sausages texture analysis is shown in Figure 3. The increase in hardness and other texture parameters is undesirable, as this effect could have a great impact on consumer acceptability.[46] Compressive strength of cooked pork sausage was higher in samples containing chitosan, which showed also higher hardness values than control samples without chitosan (Figure 3a). Lin & Chao,[25] Garcia *et al.*[67] and López-Caballero[68] reported similar behaviors in studies on the chitosan addition in samples of pork sausages and fish patties, respectively. Kachanechai *et al.*,[56] assessing the influence of chitosan in a model with chicken salt-soluble proteins indicated that the increase in compressive forces resulted in improvement of texture; this effect is due to the fact that chitosan may act as a binder favoring the formation of a stronger gel. Although addition of chitosan increases hardness, this is a positive result since a sausage with a more stable structure can be obtained. The stabilization of a meat emulsions can be related with the water and fat holding capacity,[69] being this an ability of chitosan reported in several studies,[10,11,47] Furthermore, hardness increased with reducing of fat content and by storage time ($p < 0.05$) in all samples. These effects can be explained regarding to the moisture content, since, a loss of water was observed during storage, as described before. Others authors reported the same behavior during storage[32,46,24] and cooking.[70]

The addition of chitosan in sausages caused also an increase in gumminess values (Figure 3b) in relation to control samples. These properties showed tendency to be higher in samples with a lower fat content and a higher time of storage. Estévez *et al.*[71] and López-Caballero *et al.*[68] indicated that the result of gumminess depends on the hardness, which justifies the similar behaviour shown by these parameters. Hardness is the maximum force required to compress the sample and gumminess is the force necessary to disintegrate a semi-solid state of the sample until swallowing.[72] The parameters springiness, cohesiveness, chewiness and resilience were not significantly affected by the addition of chitosan in the samples and different percentages of fat ($p > 0.05$) (data not shown). However, storage time caused effect in resilience, which was slightly reduced ($p < 0.05$) after 15 days of storage.

Sensory Evaluation

The sensory scores obtained for pork sausages containing or not chitosan and prepared with different levels of fat are shown in Table 3 Regarding the addition of chitosan, results indicated that no significant differences ($p > 0.05$) were found for the taste due to the addition of chitosan to the products However, on appearance the sample 1B and 3B (with chitosan) was statistically different ($p < 0.05$) from the respective control samples, 1A, and 3A. This effect can be justified manly by its more intense red color (a *). Sayas-Barberá *et al.*[12] reported that burgers containing low molecular weight chitosan presented best visual appearance (pink and shiny), which is in concordance with the data here shown. Soultos *et al.*[7] also reported that sausages prepared with chitosan, scored slightly higher in appearance than the respective control sample, but statistical significance was not found, after the first 7 days of storage. Lin and Chao[25] indicated that chitosan did not cause negative effect on the flavor and no significant off-odor was noted after cooking in sausage. In general, the addition of 2% chitosan on sausages resulted in a moderate difference in appearance

compared with the controls sausages (without chitosan), being both similar regarding the taste.

The obtained results give a positive result since no differences were found among samples containing or not chitosan and taking into account that, in the literature, it is possible to found a lot of references on the perception of astringency in other food matrices treated with chitosan.[73-75] Rodríguez *et al.*[76] reported a high correlation among the astringency intensity increase when solution pH decreased. Thus, meat matrices due to their pH values near neutrality can reduce the perception of astringency of chitosan, allowing the development of functional foods without changing the taste significantly.

Experimental

Sausages ingredients and chitosan

The raw meat (pork meat and fat), ingredients (salt, fresh garlic, powder white pepper and dried oregano) and artificial casings were obtained in local markets in the city of Porto (Portugal). Low Molecular Weight Chitosan (LMWC, Sigma-Aldrich, Steinheim, Germany), previously characterized[26], with a Molecular Weight (MW) of 123 KDa and 90% deacetylated, was used in the study

Fresh sausages manufacture

An equilibrated fresh sausage formulation was previously designed and consisted of 77% (w/w) of minced pork meat, 10% (v/w) of water, 1.5% (w/w) of salt, 1.3% (w/w) of fresh garlic, 0.2% (w/w) of powder white pepper and 0.1% (w/w) of dried oregano.

The chitosan content was selected in order to be sufficiently high to meet the requirements of the recently passed EFSA health claim (3 g/day). So, chitosan was added at a concentration of 2% (w/w) in order to assure that in one serving of sausages (150 g, corresponding to 3 sausages of ca. 50 g containing 1 g of chitosan each) the consumption

of 3 g of chitosan is assured. Namely, an intake of these sausages, according to the nutritional recommendation, goes along with a chitosan intake that is sufficiently high to have health promoting effects.

Pork fat was added at different concentrations: 5% (w/w) (Formulation 1B), 12.5% (w/w) (Formulation 2B) and 20% (w/w) (Formulation 3B). A sample without chitosan was used as a control for each formulation (1A, 2A and 3A). Preparation of sausages was carried out following typical procedures for the preparation of this kind of product. Thus, minced pork meat was mixed with the corresponding amount of fat. Then, the rest of ingredients were added consecutively one by one, being chitosan added in the last place. All the ingredients were fully homogenized manually for 5-10 min. After homogenization, the mixture was embedded in artificial casings obtaining fresh sausages with 3 cm of diameter and 50 g per unit. Pork sausages were packed in plastic bags without vacuum and stored under refrigeration at 4 °C for 15 days. One lot of 1000 g of fresh pork sausage of each formulation was prepared and divided into two replicates, which were analyzed in duplicate.

Microbiological analysis

With the objective to evaluate the microbiological quality throughout the storage time of the fresh pork sausages, mesophilic and psychrophilic bacteria as well as *Enterobacteriaceae* and yeast and molds counts were analysed at 0, 5, 10 and 15 days of storage at 4 ºC. Thus, 8 g of sample in 80 mL of sterile peptone water were placed in plastic bags and homogenized for 2 min in a stomacher (Lab Blender 400, London, UK). The homogenate was serially diluted with sterile peptone water and viable counts were assessed by the drop method (20 µl of each dilution), as described by Miles *et al.*[27] except for *Enterobacteriaceae*, where pour plate technique was used. Specific medium and incubation conditions for each microorganism were used. Thus, plate count agar (PCA,

Biokar diagnostics) was used for mesophilic and psychrophilic bacteria, and plates were incubated at 30 °C for 48 h and 7 °C for 7 days, respectively. Yeasts and molds were grown on Potato Dextrose Agar (PDA, Biokar diagnostics) being the plates incubated at 25 °C for 5 days. *Enterobacteriaceae* were growth in Violet Red Bile Glucose Agar (VRBGA, Lab) and the corresponding plates incubated at 37 °C for 24 h.

After incubation, the colonies were enumerated and the colony forming units (CFU/mL) were calculated.

Physicochemical analyses

Moisture, protein, fat, ash and pH. Moisture, ash, protein, and fat content of samples were determined, in raw sausages, at 0, 5, 10 and 15 days of storage at 4 ºC by the official AOAC methods of analysis 24.003, 24.009, 24.027, and 24.005, respectively.[28] In brief, the methodology is described as follows:

Moisture (g water/100 g sample) was determined by drying the samples at 105ºC to constant weight. Ash content (g ash/100 g sample) was calculated after incineration of the samples in a muffle at 550 °C and weighed. Protein (expressed as g protein/100 g sample) was analyzed by the Kjeldahl method. Fat content (g fat/100 g sample) was calculated by weight loss after extraction with hexane in a Soxhlet apparatus.

The pH values of samples were also measured by an AOC method of analysis. Specifically they were analyzed by the 943.02 method.[29] A combined pH glass electrode connected to a pH-meter MicropH 2001 Crison potentiometer (MicropH 2001, Barcelona, Spain) was used.

Lipid oxidation. Lipid oxidation was assessed by measuring the thiobarbituric acid reactive substances (TBARS) by the method adapted from.[30] Thus, 2 g of sample were homogenized by vortexing in 10 mL of 10% (v/v) of tricloroacetic acid (TCA biochemical/Applichem) and 5 ml of 0.02 M 2-thiobarbituric acid (TBA, Merck). Then, it

was centrifuged at 5000 rpm for 20 min in a Universal 320R centrifugue (Zentrifugem, HETTICH). The supernatant was collected and filtered, heated in boiling water for 35 min at 100 °C and chilled in iced water for 10 min. Finally, absorbance at 532 nm was measured in a spectrophotometer UV mini 1240 (Shimadzu, Tokyo, Japan). 1,1,3,3 tetraethoxypropane (Sigma Aldrich) was used as standard in the range $1x10^{-6}$ – $14x10^{-6}$ mol/L. TBARS concentration was expressed as mg malondialdehyde per kg of sample. Each replicated of fresh pork sausages was analysed in duplicate.

Color analysis. Color of each kind of sausage samples was determined according to the methodology described by Abularach *et al.*,[31] using a digital Minolta colorimeter (Model CR-300, Minolta, Osaka, Japan). The parameters lightness (L*), redness/greenness (a*) and yellowness/blueness (b*) were determined under the conditions indicated below, according to the specifications of the Commision Internationale de L'éclairage (CIE, 1986), being: illuminant D65, 8° viewing angle and standard observer angle of 10° specular included. Determinations in each replicated of fresh pork sausages samples were performed in triplicate.

Analysis on cooked samples

It is important to highlight that sausages are consumed after cooking and that some parameters in cooked products permit to understand how chitosan incorporation is affecting the sausages. Since chitosan impacts especially the fat and water features, moisture retention and some textural parameters were analyzed in cooked samples.

Moisture retention after cooking

Estimation of moisture retention in the sausage samples were determined according the methodology describe by Sayas-Barberá *et al.*[12] Thus, the sausages were cooked in an oven at 150ºC to a core temperature of 72 º C. This internal value of temperature was determined at the geometrical center of the samples by inserting a thermocouple. After

cooking, sausages maintained at room temperature until cooling. Samples were weighed and measured before and after cooking. The estimation the amount of moisture retained in the samples was calculates according the following equation (1):

$$\% \text{ Moisture retention} = 100 \times \frac{\text{cooked weight (g)} \times \% \text{ moisture in cooked sample}}{\text{raw weight (g)} \times \% \text{ moisture in raw sample}} \quad (1)$$

Texture profile analysis (TPA)

Fresh sausages of each formulation were subjected to cooking after 0, 5, 10 and 15 days of storage at 4ºC and analysed, in terms of texture. This analysis was carried out in a texture analyzer TA-XT2 (Stable Micro Systems, Haslemere, England). Samples were cut into pieces of 3 cm high. Textural parameters were measured by compressing the samples to 25% of their original height between flat paltes and a cylindrical probe with a cylinder probe of 2 cm of diameter. Force-time curves were recorded at a crosshead speed of 5 mm/s at a distance of 35 mm.[32] Hardness (peak force of first compression cycle, N), chewiness (hardness × cohesiveness × springiness, N x mm), cohesiveness (ratio of positive areas of second cycle to area of first cycle, dimensionless), gumminess (hardness × cohesiveness, N), springiness (distance of the detected height of the product on the second compression divided by the original compression distance, mm/mm) and resilience (area during the withdrawal of the first compression divided by the area of the first compression) were the textural parameters determined.[33] Two units of each formulation was analysed in duplicate.

Sensory Analysis

For sensory evaluation, performed only on day 0, the sausages were subjected to 130 °C for about 35 minutes in an oven to reach an internal temperature of 72 °C, and then were

subsequently cut into 5 cm pieces to be served immediately[34]. The sensory panel was composed of nine trained panelists selected from graduate students of the School of Biotechnology, Catholic University of Portugal in Porto. The cooked pork sausages were evaluated for appearance and taste, separately for each level of fat content. A difference from control test was used. Samples without chitosan were used as control samples. Each panelist received a labeled control sample, a blind control sample plus a test sample. Blind control and test samples were coded with three-digit random numbers, and presented to panelist in a balanced order. Each panelist was asked to rate the difference between the coded samples and the labeled control using the provided scale: 0 - same / no difference; 3 - moderate difference and 5 - big difference.

All experiments were performed in accordance with the relevant laws and guidelines for sensory testing of food products. All food ingredients were obtained via commercial suppliers and all additives were food-grade. Preparation prior to testing was performed in a dedicated preparation kitchen by trained food technologists. Sensory evaluation experiments that do not involve testing on under 16 year olds or the inclusion of alcohol are approved by the Board of Directors of the CBQF Research Centre. Informed written consent was obtained prior to the experiment.

Statistical analysis

The statistical package used was the Assistat software, version 7.6 beta[35] to explore the statistical significance of the results. All data were evaluated by analysis of variance (ANOVA), considering a confidence interval at the 95% level ($p < 0.05$). For the results of physicochemical and microbiological analysis was used the Tukey test with three factors: storage time, level of fat and chitosan. Data collected for the sensory analysis was evaluated by a non-parametric test for paired samples using the Wilcoxon signed-rank test.

Conclusions

The results here obtained indicated that incorporation of 2% chitosan (corresponding to 1 g chitosan/ sausage) in pork sausages to assure the ingestion of 3 g of chitosan per day (3 sausages) and to accomplish, thus, the EFSA claims of reduction of cholesterol, is technologically feasible and also allows to obtain a product with improved properties, namely if fat reduction is sought. Besides the functional value, the results also indicate that chitosan possesses an interesting potential to be included in fresh pork sausages, since it cause an increase on the stability and shelf-life of the product, considering the reduction of microbial growth and lipid oxidation. It also promote a best red color, a more stable emulsion, by the ability to bind water and fat, and a firmer texture by increase of compressive forces, without negatively affecting the sensory properties. Thus, the results here indicate that although it is necessary to conduct further studies, the addition of chitosan, besides the generation of a functional product with health claim, can act positively on the quality and shelf-life of pork sausages, and permit efficiently the reduction of the fat content.

Acknowledgements

This work was supported by National Funds from FCT through project PEst-OE/EQB/LA0016/2013. D.S. do Amaral thanks PDSE of Coordination for the Improvement of Higher Education Personnel – CAPES, Brazil (BEX 18512-12-7) for a grant under the PhD program abroad sandwich. A Cardelle-Cobas is grateful to the FCT (Fundação para a Ciência e a Tecnologia) for the postdoctoral fellowship with reference SFRH/BPD/90069/2012.

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TABLES

Table 1 Microbial counts (Log CFU/g) obtained for fresh pork sausages with 2% (w/w) of chitosan (1, 2 and 3B) and without chitosan (1, 2 and 3A) prepared with different amounts of fat, 5% (w/w) (samples 1), 12.5% (w/w) (samples 2) and 20% (w/w) (samples 3) and stored at 4 °C for 15 days

Microorganisms	Samples	Storage period (days)*			
		0	5	10	15
Mesophilic (Log CFU/g)	1ª	8.25 ± 0.03^{cA}	8.32 ± 0.03^{bcA}	8.41 ± 0.02^{bA}	8.79 ± 0.07^{aA}
	1B	7.82 ± 0.10^{bB}	7.81 ± 0.09^{bC}	7.91 ± 0.06^{abC}	8.00 ± 0.04^{aD}
	2ª	7.17 ± 0.04^{dC}	8.09 ± 0.07^{cB}	8.20 ± 0.04^{bB}	8.44 ± 0.03^{aB}
	2B	6.80 ± 0.09^{cE}	7.07 ± 0.05^{bD}	7.34 ± 0.03^{aD}	7.38 ± 0.03^{aE}
	3ª	7.11 ± 0.03^{cC}	8.14 ± 0.04^{bB}	8.23 ± 0.04^{abB}	8.25 ± 0.02^{aC}
	3B	6.91 ± 0.06^{cD}	7.18 ± 0.07^{bD}	7.14 ± 0.10^{bE}	7.45 ± 0.01^{aE}
Psychrophilic (Log CFU/g)	1ª	5.35 ± 0.02^{dA}	6.35 ± 0.03^{cA}	7.50 ± 0.03^{bA}	8.44 ± 0.01^{aA}
	1B	5.00 ± 0.07^{dC}	5.37 ± 0.02^{cD}	7.21 ± 0.02^{bC}	7.95 ± 0.04^{aD}
	2ª	5.23 ± 0.03^{dB}	6.13 ± 0.04^{cB}	7.3 ± 0.02^{bB}	8.35 ± 0.02^{aB}
	2B	4.95 ± 0.06^{dC}	5.10 ± 0.04^{cE}	7.04 ± 004^{bD}	7.86 ± 0.04^{aE}
	3ª	5.16 ± 0.04^{dB}	5.53 ± 0.01^{cC}	7.20 ± 0.02^{bC}	8.18 ± 0.04^{aC}
	3B	4.81 ± 0.06^{dD}	5.01 ± 0.04^{cF}	6.89 ± 0.05^{bE}	7.79 ± 0.07^{aE}
Enterobactereaceae (Log CFU/g)	1ª	2.80 ± 0.03^{dA}	2.94 ± 0.01^{cA}	3.87 ± 0.01^{bA}	4.65 ± 0.05^{aA}
	1B	2.64 ± 0.03^{dB}	2.72 ± 0.01^{cC}	3.60 ± 0.05^{bC}	3.80 ± 0.02^{aC}
	2ª	2.77 ± 0.04^{dA}	2.90 ± 0.02^{cA}	3.84 ± 0.03^{bA}	4.57 ± 0.03^{aB}
	2B	2.54 ± 0.06^{dC}	2.66 ± 0.02^{cCD}	3.55 ± 0.02^{bCD}	3.69 ± 0.03^{aD}
	3ª	2.75 ± 0.01^{dA}	2.82 ± 0.01^{cB}	3.75 ± 0.03^{bB}	4.59 ± 0.03^{aAB}
	3B	2.56 ± 0.04^{cC}	2.59 ± 0.04^{cD}	3.48 ± 0.02^{bD}	3.73 ± 0.04^{aD}
Moulds and Yeasts (Log CFU/g)	1ª	5.27 ± 0.03^{dA}	6.34 ± 0.04^{cA}	6.45 ± 0.03^{bA}	7.35 ± 0.03^{aA}
	1B	4.90 ± 0.09^{cC}	5.99 ± 0.05^{bC}	6.09 ± 0.04^{bC}	6.95 ± 0.04^{aC}
	2ª	5.16 ± 0.03^{cAB}	6.29 ± 0.06^{bA}	6.39 ± 0.04^{bA}	7.24 ± 0.05^{aAB}
	2B	4.85 ± 0.12^{dC}	5.84 ± 0.05^{cCD}	6.06 ± 0.03^{bC}	6.87 ± 0.10^{aC}
	3ª	5.10 ± 0.05^{dB}	6.12 ± 0.03^{cB}	6.25 ± 0.02^{bB}	7.21 ± 0.05^{aB}
	3B	4.92 ± 0.10^{dC}	5.89 ± 0.08^{cD}	6.01 ± 0.04^{bC}	6.80 ± 0.07^{aD}

*Different letters (a–b) in the same row differ significantly ($P < 0.05$) in time. Different letters (A–B) in the same column differ significantly ($P < 0.05$) in samples.

Table 2 Proximate composition obtained for fresh pork sausages and water retention capacity (WRC) calculated for raw pork sausages with 2% (w/w) of chitosan (1, 2 and 3B) and without chitosan (1, 2 and 3A) prepared with different amounts of fat, 5% (w/w) (samples 1), 12.5% (w/w) (samples 2) and 20% (w/w) (samples 3) and stored at 4 °C for 15 days

Variables	Samples	Storage period (days)*			
		0	5	10	15
Moisture (g/100g)	1A	73.10 ± 0.12^{aA}	72.21 ± 0.05^{bA}	72.13 ± 0.25^{bA}	71.73 ± 0.19^{bA}
	1B	71.54 ± 0.21^{aB}	71.17 ± 0.38^{aB}	71.39 ± 0.26^{aB}	70.35 ± 0.09^{bB}
	2ª	69.28 ± 0.16^{aC}	69.76 ± 0.16^{aC}	68.32 ± 0.30^{bC}	66.56 ± 0.08^{cC}
	2B	67.21 ± 0.23^{aD}	66.16 ± 0.17^{bD}	66.31 ± 0.19^{bD}	63.90 ± 0.16^{cD}
	3ª	67.10 ± 0.01^{aD}	64.15 ± 0.48^{bF}	61.95 ± 0.22^{dF}	62.81 ± 0.32^{cE}
	3B	66.05 ± 0.33^{aE}	64.74 ± 0.37^{bE}	62.73 ± 0.27^{cE}	61.21 ± 0.18^{dF}
Proteins (g/100g)	1ª	20.09 ± 0.10^{aA}	19.13 ± 0.03^{bA}	19.64 ± 0.25^{abA}	19.43 ± 0.27^{abA}
	1B	19.57 ± 0.03^{aA}	18.87 ± 0.14^{abA}	18.48 ± 0.80^{bB}	18.49 ± 0.01^{bA}
	2ª	19.05 ± 0.03^{aB}	18.82 ± 0.22^{aA}	18.41 ± 0.24^{aB}	18.55 ± 0.14^{aA}
	2B	19.25 ± 0.10^{aAB}	18.47 ± 0.27^{abA}	18.30 ± 0.65^{bB}	17.17 ± 0.36^{cB}
	3ª	16.34 ± 0.38^{aC}	16.02 ± 0.07^{aB}	15.83 ± 0.34^{abC}	15.08 ± 0.11^{bC}
	3B	17.00 ± 0.46^{aC}	15.64 ± 0.10^{bB}	15.24 ± 0.03^{bcC}	14.55 ± 0.36^{cC}
Fat (g/100g)	1ª	4.76 ± 0.16^{bE}	5.10 ± 0.18^{abE}	5.31 ± 0.09^{abE}	6.08 ± 0.17^{aE}
	1B	3.58 ± 0.09^{bF}	4.30 ± 0.36^{abE}	4.29 ± 0.12^{abE}	4.88 ± 0.59^{aF}
	2ª	10.52 ± 0.08^{cC}	10.85 ± 0.26^{cC}	12.15 ± 0.35^{bC}	13.19 ± 0.82^{aC}
	2B	9.28 ± 0.71^{bD}	9.52 ± 0.01^{bD}	10.60 ± 0.01^{aD}	11.12 ± 0.10^{aD}
	3ª	18.75 ± 0.23^{bA}	19.16 ± 0.55^{bA}	20.74 ± 0.65^{aA}	21.53 ± 0.54^{aA}
	3B	17.38 ± 0.28^{bB}	17.33 ± 0.04^{bB}	17.75 ± 0.19^{bB}	19.24 ± 0.51^{aB}
Ash (g/100g)	1ª	2.27 ± 0.04^{bA}	2.27 ± 0.01^{bA}	2.40 ± 0.03^{bA}	2.71 ± 0.27^{aA}
	1B	1.97 ± 0.03^{cB}	2.15 ± 0.03^{bcAB}	2.20 ± 0.02^{bAB}	2.48 ± 0.05^{aA}
	2ª	1.93 ± 0.07^{bBC}	2.08 ± 0.09^{abAB}	2.00 ± 0.04^{abBC}	2.20 ± 0.02^{aB}
	2B	1.76 ± 0.15^{bBC}	1.99 ± 0.06^{aB}	2.11 ± 0.12^{aB}	2.16 ± 0.02^{aB}
	3ª	1.70 ± 0.05^{bC}	1.71 ± 0.04^{bC}	2.00 ± 0.04^{aBC}	2.01 ± 0.03^{aB}
	3B	1.88 ± 0.13^{bBC}	1.95 ± 0.05^{abB}	1.82 ± 0.06^{bC}	2.09 ± 0.23^{aB}
WRC (%)	1ª	78.59 ± 0.03^{cE}	81.67 ± 0.46^{bF}	84.21 ± 0.46^{aE}	84.19 ± 0.24^{aE}
	1B	$96.96 \pm 0.31b^{cC}$	96.07 ± 0.70^{cD}	97.93 ± 0.28^{bC}	100.04 ± 0.13^{aC}
	2ª	91.55 ± 0.40^{cD}	89.46 ± 0.67^{dE}	95.81 ± 0.36^{bD}	97.83 ± 0.11^{aD}
	2B	107.59 ± 0.33^{cB}	109.60 ± 0.06^{bC}	111.26 ± 0.01^{abB}	112.9 ± 0.31^{aB}
	3ª	97.86 ± 0.43^{cC}	116.59 ± 0.20^{aB}	112.5 ± 0.23^{bB}	112.96 ± 0.62^{bB}
	3B	120.19 ± 0.82^{cA}	119.01 ± 0.58^{cA}	123.30 ± 0.29^{bA}	126.34 ± 0.20^{aA}

*Different letters (a–b) in the same row differ significantly ($P < 0.05$) in time. Different letters (A–B) in the same column differ significantly ($P < 0.05$) in samples.

694 **Table 3** Sensory evaluation obtained for fresh pork sausages with 2% of chitosan (1, 2 and
695 3B) and without chitosan (1, 2 and 3A) prepared with different amounts of fat, 5% (w/w)
696 (samples 1), 12.5% (w/w) (samples 2) and 20% (w/w) (samples 3)

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Samples	Appearance*	Taste*
1A	0.78^{b}	0.56^{a}
1B	2.56^{a}	1.78^{a}
2A	0.89^{b}	1.22^{a}
2B	1.56^{a}	2.11^{a}
3A	0.78^{b}	1.33^{a}
3B	2.33^{a}	2.22^{a}

698 *Paired comparisons performed within the same fat content level.
699 Different superscripts indicate no significant differences ($p>0.05$).
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LEGENDS OF FIGURES

Figure 1 Evaluation of lipid oxidation in fresh pork sausages with 2% (w/w) of chitosan (samples B) and without chitosan (samples A) prepared with different amounts of fat, 5% (w/w) (samples 1), 12.5% (w/w) (samples 2) and 20% (w/w) (samples 3) and stored at 4 °C for 15 days.

Figure 2 Evaluation of **(a)** lightness (L*), **(b)** redness/greenness (a*) and **(c)** yellowness/blueness (b*) in fresh pork sausages with 2% (w/w) of chitosan (samples B) and without chitosan (samples A) prepared with different amounts of fat, 5% (w/w) (samples 1), 12.5% (w/w) (samples 2) and 20% (w/w) (samples 3) and stored at 4 °C for 15 days.

Figure 3 Evaluation of **(a)** Hardness (N) and **(b)** Gumminess (N) in fresh pork sausages with 2% (w/w) of chitosan (samples B) and without chitosan (samples A) prepared with different amounts of fat, 5% (w/w) (samples 1), 12.5% (w/w) (samples 2) and 20% (w/w) (samples 3) and stored at 4 °C for 15 days.

FIGURES

Figure 1

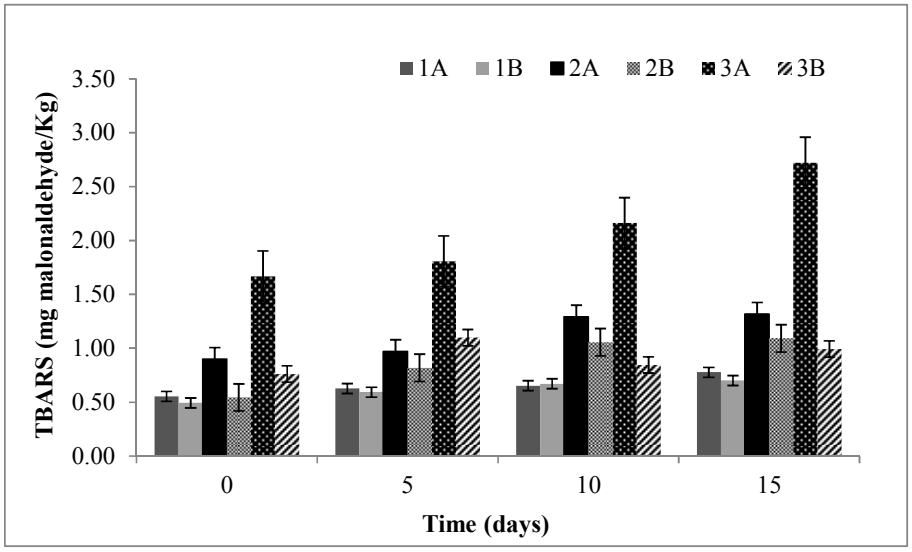

Figure 2

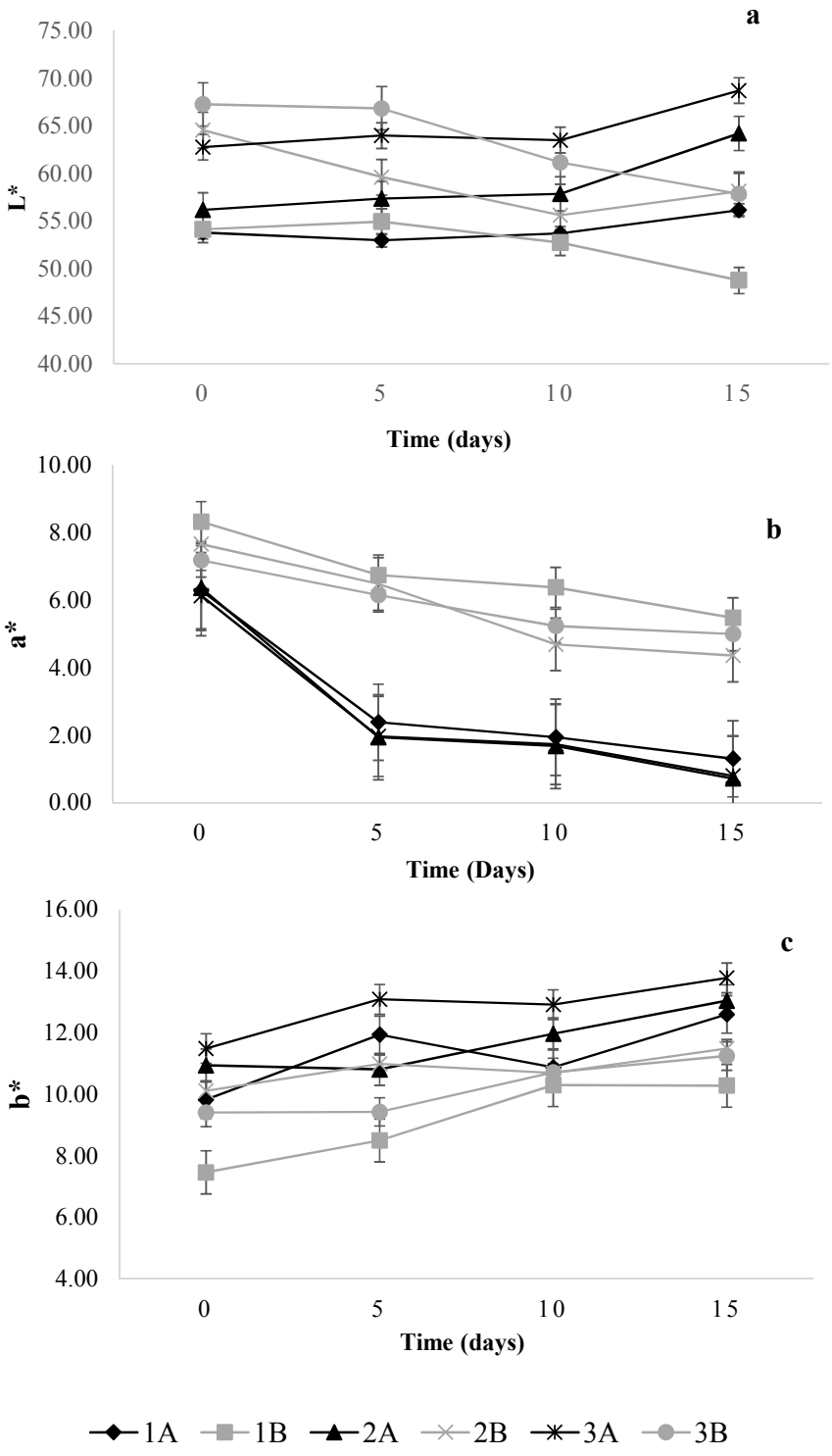

Figure 3