EFFECT OF TOCOPHEROL EXTRACT, *STAPHYLOCOCCUS CARNOSUS* CULTURE AND CELERY CONCENTRATE ADDITION ON QUALITY PARAMETERS OF ORGANIC AND CONVENTIONAL DRY-CURED SAUSAGES

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ABSTRACT

The effect of the addition in the sausage mix formulation of tocopherols (200 mg/kg), a conventional starter culture with or without *Staphylococcus carnosus*, celery concentrate (CP) (0.23% and 0.46%) and two doses of nitrate (70 and 140 mg/kg expressed as $NaNO_3$) on residual nitrate and nitrite amounts, instrumental CIE Lab color, tocol content, oxidative stability and overall acceptability were studied in a fermented dry-cured sausage after ripening and after storage. Nitrate doses were provided by the nitrate rich-CP or a chemical grade source. The lower dose meets the maximum ingoing amounts in EU for organic meat products. Tocopherol addition protected against oxidation whereas either the nitrate dose, nitrate source or starter culture had no influence on secondary oxidation values. The residual nitrate and nitrite amounts found in the sausages that had the lower nitrate dose were below the allowed limits in the EU for organic meat products and residual nitrate can be even much more reduced by the presence of the *S. carnosus* culture. The low dose CP does not affect color measurements and any of the studied factors affect negatively on product consumer acceptability. The two nitrate sources behave similarly for the studied parameters and, in consequence, CP is a useful alternative to chemical ingredients for organic dry-cured sausage production.

KEYWORDS: dry-cured sausages, organic production, nitrate and nitrite reduction, celery concentrate, *Staphylococcus carnosus*

INTRODUCTION

Ancient Greeks and Romans used salt to preserve fish and meat through the curing process and this type of food products are still present in our diets. Salt was historically believed to be responsible for obtaining cured meat products, however, it has been demonstrated that nitrate impurities are crucial for curing (1).

Nowadays, it is completely understood that sausages like the Spanish *salchichón* or the Italian *salame* are dry-fermented cured meat products that require the presence of several ingredients such as salt, sweeteners, a nitrate or nitrite source as well as a bacterial culture to develop their distinctive color, flavor and texture properties (2). The addition of nitrate, which is first reduced to nitrite, or directly the addition of nitrite is completely necessary to develop the typical color and flavor, in addition, acts as an antimicrobial to control *Clostridium botulinum* and also helps to prevent oxidation (1, 3).

However, the health concern about nitrite because of the carcinogen nitrosamine formation ended up with the reduction of their content in cured meats since mid-1970s (4, 5) and with the application of regulations about the amounts of nitrate and nitrite to be added or to be found in the cured product (6). The endogenous and/or exogenous microbiota present in the sausage mix is of interest for the industry since nitrate and nitrite amounts can be reduced thanks to the action of some specific bacteria while allowing the curing process. In relation to this, many starter cultures include *Staphylococcus sp.* since the nitrate reductase activity is typically present in *Micrococcaceae* (2). The formation of nitrite can exert an antioxidative effect by preventing the release of iron from the porphyrin molecule, estabilization of unsaturated lipids within membranes against oxidation, interaction of nitrite as a metal

71 chelator, and formation of nitroso and nitrosyl compounds acting as radical scavengers (1).
72 Therefore, the reduction of nitrite levels may affect the susceptibility to oxidation thus
73 making necessary to protect these meat products with antioxidants.
74 However, most of the formed nitrite is reduced to nitric oxide either by nitrite reductase
75 activity or by chemical reactions favored by exogenous and endogenous reductants (2).
76 Myoglobin reacts with nitric oxide producing nitrosylmyoglobin, but this can be also formed
77 through an indirect way in which nitrites oxidize myoglobin into metmyoglobin and,
78 subsequently, reacts with nitric oxide thus producing nitrosylmetmyoglobin (1, 7). In the
79 presence of exogenous and endogenous reductants, nitrosylmetmyoglobin is able to
80 autoreduce to the more stable form nitrosylmioglobin (1). The formation of this heme
81 complex gives the typical color of dry-cured meat products.
82 Nowadays, there is an increase in demanding healthier and organic food products in which
83 any chemical preservative is desired. In traditional cured meat products, however, consumers
84 dislike those in which nitrite has not been added (8). As a consequence, EU regulates the
85 nitrate and nitrite maximum amounts to be added (80 mg/kg of either $NaNO_3$ or $NaNO_2$) and
86 the maximum residual amounts to be found (50 mg/kg of either $NaNO_3$ or $NaNO_2$) in organic
87 meat foods (9). An alternative to the addition of chemical grade nitrates or nitrites is the
88 addition of natural sources containing these compounds thus acting like in those ancient times
89 when former curing salts contained nitrate impurities necessary for the curing process. In this
90 frame, some vegetable sources such as celery powder concentrates (CP) are known to be rich
91 in nitrate (10) so they can be used as substitutes of the chemical addition of nitrate or nitrite
92 when making cured sausages (11, 12).

93 The aim of this work is to assess the possibility to reduce nitrate and nitrite amounts by
94 studying how are affected different quality parameters of sausages produced under different
95 conventional and organic strategies.
96
97 **MATERIAL AND METHODS**
98
99 **Reagents and Standards**
100 Tocopherol extract (Guardian™, 70% of mixed tocopherols) was from DANISCO
101 (Copenhagen, Denmark). Conventional starter culture containing *Lactobacillus sakei* and
102 *Staphyloccocus xylosus* (SM-181 Bactoferm™), a nitrate reductase-active culture containing
103 *Staphylococcus carnosus* (CS 299 Bactoferm™) and CP were from CHR Hansen (Hørsholm,
104 Denmark). Potassium nitrate (Suprapur®), cadmium (coarse powder), copper(II) sulfate
105 pentahydrate, sodium nitrite and N-1-napthylethylenediamine dihydrochloride (NED) were
106 from Merck (Darmstadt, Germany). Sulphanilamide was from Carlo Erba (Milano, Italy).
107 Sodium ascorbate, dextrose and lactose were from Espècies Teixidor (Manresa, Spain).
108 Tocopherol analogs standard was from Calbiochem (San Diego, CA). All chemicals used
109 were of ACS grade with the exception of the solvents used in induced ferrous oxidation-
110 xylenol orange (FOX) method and the tocols determination that were of HPLC grade.
111
112 **Experimental Design**
113
114 Sixteen treatments resulted from a 2x2x4 factorial design (Table 1) planned to study the
115 influence of the addition of a tocopherol extract in the sausage mix formula (0 and 200 mg of

116 mixed tocopherols/kg meat), two starter cultures (conventional and conventional plus
117 *Staphylococcus carnosus*) and 4 different sources of nitrate (either KNO_3 or CP sources
118 providing 70 and 140 mg of nitrate/kg expressed as $NaNO_3$) on several cured meat quality
119 parameters. These two nitrate levels were chosen according to the maximum level of ingoing
120 sodium nitrate allowed in meat products (6, 9). Meat with the ingredients was stuffed into
121 natural casing and allowed to be dry-cured for 48 days. Finally, the storage time factor of the
122 dry-cured sausage, sliced and packed in modified atmosphere 0 or 45 days, was added to this
123 design thus resulting in 32 treatments.
124
125 **Sausage Preparation**
126 A meat mix consisting of 91.7% diced pork meat plus 8.3% of diced back fat from organic
127 pigs was used to prepare the ground meat. After homogenization, the raw mix batter was then
128 divided in 2 sets of 24 kg. Subsequently, the following common ingredients were added to
129 each set: 0.5 g/kg of sodium ascorbate, 3 g/kg of dextrose, 5 g/kg of lactose, 3 g/kg ground
130 black pepper, 22 g/kg of salt and 0.25 g/kg of a conventional starter culture. Natural spring
131 water was used to deliver the sodium ascorbate (100 mL) and the starter culture (100 mL)
132 into the mix. In each set, 100 mL of sunflower oil with or without tocopherol extract
133 supplementation were added. After the addition of all these ingredients samples were mixed
134 during 4 min. Samples from these 2 mixes, with and without tocopherol extract, were finely
135 ground and vacuum-packed in high-barrier multilayer bags (Cryovac BB325; approximately
136 20 g of meat/bag) and stored at -25 °C until analysis.
137 Each mix batter set was divided again into two more subsets resulting in four different groups
138 of 12 kg of meat in which 100 mL of natural spring water with or without the *Staphylococcus*

carnosus culture (1.33 g) were added according to the experimental design (Table 1). The resulting mixes were homogenized during 2 min. According to the experimental design mixes were subdivided in 16 batters of 3 kg in which CP (added at the following amounts: 6.9 g or 13.8 g) or chemically pure KNO_3 (>99.99%) (added at the following amounts: 253 mg or 506 mg) were added previously being all dissolved in 50 mL of double deionized water. These amounts were added to provide, respectively, the doses of 70 and 140 mg of nitrate/kg expressed as $NaNO_3$. The resulting 16 different raw mix batters were mixed manually during 2 min and stuffed into natural casings (40-45 mm diameter). In order to check the nitrate dosage, samples from these 16 mix batters were finely ground and vacuum-packed in high-barrier multilayer bags (Cryovac BB325; approximately 20 g of meat/bag) and stored at -25 °C until nitrate and nitrite analysis. Also, samples from these mix batters were aseptically taken for microbiological analysis and stored at 4 C until the analysis which was initiated within the same day.

Sausage Dry-Curing and Sample Preparation

Sausages were hanged for 48 days in a ripening chamber at 14±2 C and 75-85% moisture. At this time (storage time 0) half of the sausages were ground and vacuum-packed in high-barrier multilayer bags (Cryovac BB325; 180 x 200 mm; permeability to oxygen, 25 $cm^3 \times m^{-2} \times day^{-1} \times bar^{-1}$ at 23 C and 0% RH, DIN 53380; approximately 20 g of meat/bag) and stored at -25 °C until analysis. The remaining sausages were sliced (2 mm thickness) and packaged in sealed metallized polyester/polyethylene bags [Termopack PETM/PE; 300 x 200 mm; permeability to oxygen, nitrogen and carbon dioxide was respectively 50, 10 and 150 $cm^3 \times m^{-2} \times day^{-1} \times bar^{-1}$ at 23 C and 0% RH, DIN 53380; approximately 20 slices/bag] containing

80% N_2 and 20% CO_2 during 45 days at 4 C (storage time 45). After this period samples were ground, vacuum-packed in high-barrier multilayer bags and stored until analysis as done at time 0. For the microbiological analysis, samples were aseptically taken at the different storage times, 0 and 45 days, and stored at 4 C before analysis which was initiated within the same day.

Moisture Determination

The ISO 1442 procedure (13) was used to determine the moisture of the samples. Those results expressed as dry matter basis were calculated taking into account the sample moisture.

Determination of Crude Fat Content and Fatty Acid Composition

The fat content of the raw mix batters was measured according to AOAC Official Method 991.36 (14) whereas the fatty acid composition was as described elsewhere (15). Fat content was expressed in fresh weight basis whereas fatty acid composition was expressed as area normalization in percent.

Nitrite and Nitrate Determination

Ten g of sample were weighed in a 250 mL beaker and approximately 80 mL of distilled water were added. Then, Carrez I and Carrez II solutions (3 mL each) were added and the solution was filled up to 100 g. Subsequently, this solution was homogenized using a high speed homogenizer (Ultraturrax T25 basic with a dispersing tool S25N-18G, IKA-Werke GmbH, Germany) at 3500 rpm for 75 sec. The homogenate was then centrifuged at 4350 *g* for 20 min and the supernatant was used for nitrate and nitrite analyses.

185 Nitrate and nitrite analyses were performed on a segmented continuous flow system
186 (AutoAnalyzer 3 model, SEAL Analytical, UK). However, each determination was carried
187 out in a different analytical unit. Nitrate content of the clarified samples was reduced to nitrite
188 using a copperized cadmium reduction column. Subsequently, nitrite reacted with
189 sulfanilamide for diazotization and coupling with NED forms a purple azo dye. The dye
190 absorbance was then read through at 550 nm. Nitrite was determined in another analytical
191 unit using the same reaction although omitting the previous reduction step. Nitrate amounts
192 were calculated by difference. Results for raw mix batter were expressed as $NaNO_3$ or
193 $NaNO_2$ per kg of in fresh weight basis whereas results for sausage were expressed as mg of
194 $NaNO_3$ or $NaNO_2$ per kg in dry weight basis.
195
196 **Microbiological Analyses**
197 Twenty five grams of either raw batter or sausage samples, the latter previously diced in
198 small pieces, were aseptically taken and homogenized with 75 mL of buffered peptone water
199 (BPW; OXOID, Basingstoke, UK) for 2 min in an IUL masticator (IUL S.A., Barcelona,
200 Spain). Serial decimal dilutions were made in sterile Ringer ¼ solution (Scharlau, Barcelona,
201 Spain). The following foodborne pathogens were determined in raw sausages. *Escherichia*
202 *coli* were enumerated on McConkey agar (OXOID, Basingstoke, UK) and the population of
203 sulfite-reducing clostridia by counting in SPS agar (Scharlau, Barcelona, Spain) anaerobically,
204 both agars were incubated at 37 C for 48 h. The absence of *Salmonella* was determined by
205 preenrichment in BPW 16 h at 37 C, enrichment in Selenite Cystine broth (OXOID,
206 Basingstoke, UK) 24 h at 37 C and Rappaport Vassiliadis broth (OXOID, Basingstoke, UK)
207 24 h at 42 C, and isolation on SS agar (OXOID, Basingstoke, UK) and DCLS agar (OXOID,

208 Basingstoke, UK) both agars were incubated 48 h at 37 C. Kligler Iron agar (OXOID,
209 Basingstoke, UK), Lysine Iron agar (OXOID, Basingstoke, UK), Urease broth (OXOID,
210 Basingstoke, UK) and API 20E® system (bioMérieux España, Madrid, Spain) were used to
211 identify colonies grown on SS agar and/or DCLS agar. Starter bacteria were analyzed by
212 spread plating on MRS agar (OXOID, Basingstoke, UK) for lactic acid bacteria and on
213 Mannitol Salt agar (Cultimed, Barcelona, Spain) for staphylococci, both cultures were
214 incubated at 30 C for 3 days. Results were expressed in dry weight basis.
215
216 **Color Measurements**
217 Color measurements were conducted using a Konica Minolta Chroma-meter (model CR-410;
218 Konica Minolta Sensing, Inc., Osaka, Japan) based on the CIE L*a*b* color space. CIE
219 (Commission International de L'Eclairage) lightness "L*", redness "a*", and yellowness "b*"
220 values were determined from four random different surfaces of the ground samples. The
221 instrument was set for illuminant D-65 and 10º observer angle, and standardized using a
222 white standard plate.
223
224 **Content in Lipid Hydroperoxides and Susceptibility to Oxidation**
225 As reviewed elsewhere the FOX method measures lipid hydroperoxides (16). However, the
226 same method can be used to determine the existing content in these primary oxidation
227 compounds when it is measured after 30 min of incubation and also to determine the
228 susceptibility to oxidation when the method is carried out over longer periods of incubation
229 then working as an induced method (16).

Both the content in lipid hydroperoxides and the susceptibility to oxidation of the sausage samples can be determined by carrying out the same assay. Briefly, 2 g of sample were mixed and homogenized with cold methanol. Extracts were added to an acid ferrous medium containing xylenol orange. The measurement after 30 min of incubation at room temperature was used to determine the lipid hydroperoxide content (LHPC) expressed as mmol cumene hydroperoxide (CHP) eq /kg in dry weigh basis. The time course of lipid hydroperoxides formed after incubation over a time of 210 hr was used to calculate of the induced-FOX parameters. Those parameters were maximum lipid hydroperoxide value (MAXLHP), time in which the maximum lipid hydroperoxide value was achieved (TMAX), oxidation rate (OR), lipid hydroperoxide value obtained at the end of the incubation period (Final LHP) and area under the curve (AUC), and were calculated as described elsewhere (17) with the difference that parameters were expressed in dry weight basis.

TBA Determination

The TBA values of samples were determined through third-derivative spectrophotometry after acid aqueous extraction (18). Results were expressed as μg of malondialdehyde per kg of sausage in dry weight basis.

Tocopherol and Tocotrienol Analogs Determination

Tocopherol and tocotrienol analogs were determined as described elsewhere (19). Results for raw mix batter as mg of each tocol per kg in fresh weight basis whereas results for sausages were expressed as mg of each tocol per kg in dry weight basis.

253 **Sensory Analyses**

254 The following different tests were carried out:

255 *Overall Acceptability*. The 16 treatments were randomly presented to the consumers in a
256 balanced incomplete block design (20): 16 blocks, 6 samples per block, and 6 replicates for
257 each sample. This design was duplicated. In addition, each panelist evaluated the
258 acceptability of a blind control (total samples presented to each panelist = 7), which was the
259 treatment number 4. Panelists were asked to rank the overall acceptability of the product
260 using a 9-point scale (1 = very bad; 9 = very good). Thirty two volunteers were used to
261 evaluate the overall acceptability of the product.

262 *Color Triangle Test*. Samples of treatment 3 and 14 (see Table 1 for sausage formulation
263 factors) were used to perform this test to asses whether a difference existed in the color of the
264 samples. Twenty four panelists were used to perform this test.

265 *Color Intensity Ranking Test*. Samples of treatments 1, 2, 3, 4, 8, 12 and 16 (Table 1) were
266 randomly presented to the panelists and they were asked to rank the color intensity. Thirty
267 panelists were used to perform this test. Along with this test panelists were asked to select
268 their preferred sample.

269 In each test, several slices of sample sausages were placed in white plastic dishes, identified
270 by random three-digit numbers and served to the consumers' panel at room temperature.
271 Water and unsalted crackers were provided to panelists to cleanse their palates between
272 samples.

273

274 **Statistical Analyses**

A multifactor ANOVA was carried out to determine significant differences produced by the different factors on sample moisture, microbial determinations, nitrate and nitrite content, color measurements, tocopherol and tocotrienol analogs, TBA values, LHPC and induced FOX parameters. Factors were tocopherol addition (0 and 200 mg of tocopherol analogs/kg), starter culture (conventional and conventional plus *S. carnosus*), nitrate source (70 mg of $NaNO_3$/kg and 140 mg $NaNO_3$/kg, each dose provided by the addition of CP or chemical grade KNO_3) and time (after ripening, hereafter referred as day 0 or time 0, and after 45 days of storage under a modified atmosphere). Because microbiological determinations were carried out at three different periods (after starter culture inoculation in raw mix batter, 0 and 45 days) these periods were included for the time factor. Interactions between more than two factors were ignored. When main effects were significant, the least squares means were separated using the Scheffé's test ($\alpha = 0.05$).

As for consumer's acceptability sensory analysis, the storage time factor was not studied. The significance estimation in triangle and ranking tests was analyzed using tables (21, 22).

Spearman correlation coefficients between sausage TBA values and color measurements; and between tocopherol analogs and FOX parameters were calculated. In all cases, $P \leq 0.05$ was considered to be significant.

RESULTS

Moisture, Crude Fat Content and Fatty Acid Composition of Raw Mix Batters

The moisture average of the raw mix batters with and without the addition of the tocopherols extract are 61.86 ± 0.13 and 62.19 ± 0.07, respectively. The crude fat content average of the

298 raw mix batters with and without the addition of the tocopherols extract are 17.2 ± 0.29 and
299 16.7 ± 0.27, respectively. The relative percents of saturated fatty acids, monounsaturated fatty
300 acids and polyunsaturated fatty acids of the raw mix batter without the addition of the
301 tocopherols extract are 40.2%, 40.8% and 19.0%, respectively whereas for the raw mix batter
302 with the addition of the tocopherols extracts are 40.5%, 40.7% and 18.8%, respectively. A
303 table including all the quantified fatty acids in these mixes is available as Supporting
304 Information. These results demonstrate the homogeneity of these two mixes.
305
306 **Microbiological analyses**
307 The presence of food poisoning bacteria was checked in the 16 raw mix batter samples. *E.*
308 *coli* was less than 100 CFU/g, *Salmonella* sp. was absent in 25 g and sulfite-reducing
309 clostridia was less than 10 CFU/g for the sixteen sausages. All samples accomplish
310 microbiological standards for raw minced meat.
311 Lactobacilli and total staphylococci bacteria were analyzed in raw mix batter samples just
312 before stuffing as well as after curing (0 days) and after 45 days of storage at 4 C in sealed
313 bags under modified atmosphere (Table 2). Lactobacilli and staphylococci bacteria were only
314 affected by time. During fermentation the population of lactic acid bacteria increased at the
315 initial stages and then it began to decrease slowly along the processing of a fermented meat
316 product (2, 23) thus explaining that at time 0 the levels of *Lactobacillus* sp. were higher than
317 in raw mix batter samples. In both cases a reduction in the population during the storage time
318 was observed. These results are in agreement with those reported by Marco et al. (24). The
319 environmental conditions during storage, such as low temperature, moisture, nitrate

320 concentration and a reducing atmosphere could have enhanced the decrease of starter
321 population.
322
323 **Nitrate and Nitrite Residual Amounts**
324 Nitrate amounts in raw mix batters, expressed in fresh weight basis, averaged 127 ± 13 mg
325 NO_3 / kg and 66 ± 5.8 mg / kg for those treatments containing the higher and lower dose of
326 nitrate, respectively. Therefore, the nitrate dosage was well-done. Raw mix batters contained
327 trace amounts of nitrite which means that the detected amounts were between the
328 quantification and detection levels.
329 A significant interaction was found between nitrate source x starter culture for nitrate content
330 in sausages ($P \leq 0.001$). When the ANOVA was run again taking into account the two doses
331 instead of nitrate source no interactions were found (data not shown) whereas the dose effect
332 was significant thus indicating that nitrate source effect is also explained by the two levels of
333 nitrate.
334 The higher dose of nitrate added in the raw mix batters led to sausages with higher residual
335 nitrate content whereas no differences were observed between CP and chemical sources of
336 nitrate (Table 3). In addition, *S. carnosus* decreased the residual nitrate content in the sausage
337 because of its reported nitrate reductase activity (2). As expected, tocopherol addition had no
338 effect on residual nitrate content. Likewise, storage time under modified atmosphere did not
339 influence on the residual nitrate content Table 3).
340 Neither the different ingredients added in the raw mix batter formulation nor the storage
341 under modified atmosphere affected the residual nitrite content (Table 3).
342

343 **Sausage Color**

344 Several interactions were found between different factors for L*, a*, and b* values (Table 3).
345 When ANOVA was run again taking into account dose of nitrate added and source of nitrate
346 separately, instead of their combination, L* and b* were significantly higher with higher
347 doses and with the addition of CP, thus explaining some of those significant interactions. For
348 a* values the significant interactions always included the nitrate source and starter culture
349 factors.

350 Meat lightness (L*), redness (a*) and yellowness (b*) were increased by the addition of
351 tocopherols (Table 3). Lightness and yellowness were also increased in those sausages in
352 which the higher dose of CP had been added in the sausage formulation. In addition, the
353 presence of *S. carnosus* led to sausages with increased L*, a* and b* values whereas these
354 values were decreased after 45 days of storage under a modified atmosphere at 4 C.

355

356 **Tocol Content**

357 The tocol content was determined in those raw mix batters with and without the tocopherols
358 extract. The α-tocopherol, β-tocopherol, γ-tocopherol and α-tocotrienol content in the mix
359 without the addition of the tocopherol extract averaged 7.8 ± 0.93, 0.069 ± 0.011, 0.18 ±
360 0.015, and 0.36 ± 0.051 mg / kg expressed in fresh weight basis, respectively. On the other
361 hand, the α- tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol and α-tocotrienol in the mix
362 containing the tocopherol extract averaged 26.2 ± 2.9, 1.8 ± 0.23, 61.3 ± 2.0, 9.2 ± 0.69 and
363 0.4 ± 0.28 mg / g expressed in fresh weight basis, respectively. In both cases, those tocols
364 below the quantification limits were not reported.

365 Several interactions were found between different factors for the content of the different
366 tocopherol and tocotrienol analogs in sausages (Table 4). When ANOVA was run again
367 taking into account dose of nitrate added and source of nitrate separately, instead of their
368 combination, the dose factor was significant whereas there are no differences between CP and
369 chemical sources. This effect explains many of the interactions and, overall, high doses of
370 nitrate in the sausage provoke high amounts in the different tocopherol analogs.
371 The tocopherol extract is rich in different tocopherol analogs, especially in γ-tocopherol
372 (footnote in Table 1). Therefore, the addition of this supplement in the formulation led to
373 significant changes in the content of the different analogs found in the sausage (Table 4). The
374 β-, γ- and δ-tocotrienol analogs were normally below the quantification limits and, in
375 consequence, were not reported. The content of α-tocopherol, β-tocopherol and α-tocotrienol
376 was lower in those sausages in which the lower dose of chemical nitrate has been added.
377 Decreased content in all tocopherol analogs was observed by either the addition of the
378 conventional starter culture or storage time under modified atmosphere (Table 4). This
379 decrease over time can be related to an increased lipid oxidation during storage.
380
381 **Oxidative Status and Susceptibility to Oxidation**
382 The oxidative status was assessed by measuring primary and secondary oxidation products in
383 sausages. TBA determination measures malondialdehyde which is a typical secondary
384 oxidation product derived from lipid oxidation (25) whereas the FOX method measures lipid
385 hydroperoxides which are primary oxidation products (16). However, it should be noted that
386 this assay was used to measure the current lipid hydroperoxide content in sausages (LHPC
387 values) but it was also used to measure the lipid hydroperoxide formation over time thus

388 assessing the susceptibility of samples to oxidize (16). Various parameters, described
389 elsewhere (17), had been used when this method was used as an induced method to describe
390 the susceptibility to oxidation curve fashion.
391 Significant interactions between tocopherols x nitrate source (P = 0.006), tocopherols x
392 storage time (P = 0.007) and nitrate source x storage time (P = 0.047) were found for the lipid
393 hydroperoxide content. In addition, the ANOVA showed that the nitrate source factor was
394 significant (P = 0.020) but the differences between means were not big enough to be
395 separated. This significant effect may in part explain those recorded interactions. The
396 addition of tocopherols extracts reduced the lipid hydroperoxide content thanks to its
397 antioxidant activity (Table 5). The storage time also provoked an increase in the lipid
398 hydroperoxides.
399 As for the secondary oxidation, TBA values showed significant interactions between storage
400 time x tocopherols ($P \leq 0.001$), storage time x starter culture (P = 0.013) and starter culture x
401 nitrate source (P = 0.002). As expected, the addition of the tocopherols led to sausages with
402 lowered secondary oxidation values because of its antioxidant activity (Table 5). Lipid
403 oxidation is increased over storage thus recording higher TBA values when samples had been
404 stored for 45 days (Table 5). Neither the starter culture nor the different nitrate sources
405 showed to influence on TBA values.
406 Several significant interactions were found between the different factors for the induced-FOX
407 parameters (Table 6). When ANOVA was run again taking into account dose of nitrate added
408 and source of nitrate separately, instead of their combination, the dose was significant for
409 TMAX and OR whereas source was significant for MAXLHP, TMAX, OR and AUC.

410 Overall, higher doses of nitrate and CP addition provoked a reduction of the susceptibility to
411 oxidation. These effects explain many of the recorded interactions.
412 All the defined parameters of the susceptibility to oxidation with the exception of TMAX
413 showed significant differences because of the tocopherol addition in the sausage formula
414 (Table 6). Overall, these results indicate that tocopherols are efficient in delaying lipid
415 oxidation onset. The lower dose of chemical nitrate also showed significant differences with
416 other nitrate sources for all the defined FOX parameters whereas no differences were
417 observed between the other 3 nitrate source combinations. This suggests that the lower dose
418 of chemical nitrate is the least effective in preventing lipid oxidation. The addition of *S.*
419 *carnosus* showed increased values for MAXLHP, Final LHP and AUC thus suggesting that
420 sausages in which this culture had been added were more prone to oxidize. All the induced-
421 FOX parameters were increased after 45 days of storage under modified atmosphere at 4 C.
422 Oxidation values (Tables 5 and 6) indicate that stored sausages undergo slight oxidation
423 during this storage time.
424
425 **Sensory Characteristics**
426 In table 5, there are shown the results for the overall acceptability test carried out after 45
427 days of storage under a modified atmosphere in sealed bags. At this time, consumers were not
428 able to find significant differences between sausage formulations.
429 In addition to this sensory test, a triangle test was carried out in order to better test whether
430 existed significant differences in color between samples 3 and 14 (see Table 1 for formulation
431 factors) since these samples showed the maximum differences from the data obtained through
432 the colorimeter. In this triangle test, 14 out of 24 panelists ($P \leq 0.5$) matched the similar

433 samples. When these panelists were also asked which overall color was preferred, 11 out of
434 14 indicated that the sample without the tocopherols added in the formulation was preferred.
435 Later on, a sensory test was carried out to ask panelists to rank samples by overall color
436 intensity using sausages from the following treatments: 1, 2, 3, 4, 8, 12 and 16 (see Table 1
437 for formulation factors). Treatment 12 was ranked in the first position as the least dark,
438 followed by treatment 16 in 2^{nd} position, treatments 3 and 4 in 3^{rd} and 4^{th} positions
439 indistinctly, treatment 8 was ranked in 5^{th} position, treatment 1 in 6^{th} position and finally
440 treatment 2 was ranked in as the darkest sample. With the exception of positions 3 and 4, the
441 other positions were significant at $P \leq 0.05$. Collectively from these results it can be observed
442 that the addition of tocopherols in the formulation led to more clear sausages which is in
443 agreement with colorimeter values. Nevertheless, the addition of *S. carnosus* led to darker
444 sausages when tocopherols had not been added. Finally, according to the panelist, the
445 presence of CP in the sausage formulation led to a darker color. Along with this test, panelists
446 were also asked to indicate their preferred sample according to the appearance (among
447 panelists the 30% preferred sample 3, whereas the 23%, 17% and 13% of the panelists
448 preferred samples 12, 1 and 4, respectively) which was not related with the darkness ranking.
449

450 **DISCUSSION**

451

452 EU regulates nitrate and nitrite indicative ingoing amounts (each at 80 mg/kg expressed as
453 $NaNO_3$ or $NaNO_2$, respectively) and the maximum residual amounts (each at 50 mg/kg
454 expressed as $NaNO_3$ or $NaNO_2$, respectively) in organic meat products (9). Results showed
455 that when the dose added to the sausage raw mix batter meets the regulation (70 mg

456 $NaNO_3$/kg) the residual nitrate and nitrite amounts were far below the limits (Table 3). In
457 addition, no differences were observed in the residual content of nitrate or nitrite when
458 comparing the CP or the chemical source.
459 Nitrate is reduced to nitrite by nitrate reductase activity from bacteria that are either naturally
460 present in the raw mix batter or exogenously added. The conventional starter culture we used
461 in this study contained *Lactobacillus sakei* and *Staphylococcus xylosus*. Despite the fact that
462 *S. xylsosus* possesses nitrate and nitrite reductase activity, this is not as intense as that of *S.*
463 *carnosus* (26). Thanks to its high nitrate-reductase activity, the addition of *S. carnosus* culture
464 to the mix reduced very efficiently the nitrate amounts thus ensuring optimal color formation
465 during initial fermentation stages (11, 27). This activity seems to disappear with storage time
466 which can be due to the environmental conditions after the curing process (Table 3). The
467 formed nitrite is a reactive compound that can be further reduced after reacting with heme
468 moiety and various endogenous and exogenous reductants such as ascorbate (1, 7). This
469 reactivity can explain the recorded residual nitrite decrease after 45 days of storage.
470 Alternatively, Ahn et al. (28) found lower residual nitrite in vacuum packed sausages than
471 those stored under aerobic conditions and they attributed that the reducing environment
472 allowed the conversion of nitrite to nitric oxide.
473 Nitrite acts as a preservative inhibiting the growth of undesirable microorganisms, especially
474 *Clostridium botulinum*, but their contribution to the typical cured meat flavor and color is
475 crucial (1, 2). The nitrosylmyoglobin formed after reaction of myoglobin with nitric oxide (1)
476 is ensured at 70 mg/kg regardless of the nitrate source and, according to the colorimetric data,
477 this dose seemed to be not significantly different in color in comparison to those sausages
478 that received conventional doses (chemical 140 mg/kg) of nitrate (Table 3). These results are

479 in agreement with other works using CP in cooked ham processing (11). However, in the
480 present work, the addition of CP at high doses led to sausages with higher lightness and
481 yellowness which could be due to the intrinsic color of the concentrate powder.
482 Isabel et al. (29) found that a higher α-tocopherol concentration in dry-cured hams reduced
483 color fading and weight loss thus explaining the higher moisture content found in those
484 sausages receiving the tocopherols extract. Therefore, it is possible that the higher water
485 amounts found in those sausages enriched with tocopherols provoked the increase in L* a* b*
486 values (Table 3) since the addition of 200 mg/kg of tocopherols extract in a sunflower oil
487 matrix did not significantly increase these instrumental color values (data not shown).
488 Despite the fact that other authors reported no effect on the color stability of cured pork
489 products from animals that received diets rich in tocopherol (30, 31), the addition of this
490 extract may protect from oxidation thus maintaining color properties during fermented
491 sausage ripening. Meat discoloration because of oxidation and lipid oxidation is a major
492 drawback (32, 33) and the protective effect of tocopherols against lipid oxidation during
493 ripening is clearly observed by looking at primary and secondary oxidation values (Table 5).
494 These two oxidation parameters provided similar information since both showed increased
495 oxidation values after 45 days of storage whereas no differences were found for nitrate source
496 and starter culture factors.
497 The decrease in redness values and the increase in yellowness values are often related with
498 increased lipid oxidation. After 45 days of storage under a modified atmosphere, all color
499 parameters (L*, a* and b*) were lower in comparison to those found after ripening (Table 3).
500 Rubio et al. (34) studied the effect of storage time on color stability in a conventional dry-
501 cured sausage stored under the same modified atmosphere (20% CO_2 and 80% N_2). These

502 authors found that comparing instrumental color values after 0 and 120 days of storage, L*
503 and a* values were increased whereas yellowness was decreased. However, they also found
504 that these trends changed at different storage periods. In addition, the recorded interactions
505 between nitrate source and storage time (Table 3) could likely confound some factor effects
506 on color since a positive Spearman correlation between TBA values and yellowness (r_s =
507 0.500, P = 0.049) was found only when using the data obtained from samples stored during
508 45 days.
509 In addition to that correlation, and also using the data obtained from samples stored during 45
510 days, TBA values and redness showed a negative Spearman correlation coefficient (r_s = -
511 0.724, P = 0.002). Redness is being used as an indicator of color stability since oxidative
512 discoloration of cured meats converts nitrosylmyoglobin to nitrate and metmyoglobin (35,
513 36). This phenomenon will explain the recorded decrease in redness during storage (Table 3).
514 It has been reported that red color was more stable over a storage period and lipid oxidation
515 was lower when low nitrite cured pork products (50 mg/kg) came from animals that received
516 500 mg α-tocopheryl acetate/kg feed supplementation (37).
517 Color influence consumers decisions so these differences in sausage color may be detected by
518 consumers and, eventually, can be associated to product quality and freshness (38). In
519 relation to this, two sensory analyses were carried out in those sausages stored for 45 days to
520 evaluate whether those differences found using a colorimeter could be detected by a sensory
521 panel. Two samples having the maximum difference in instrumental color values were
522 selected for a triangle test. In this test, panelists were able to differentiate a sausage in which
523 neither tocopherols nor *S. carnosus* had been added in comparison to another sausage in
524 which both were added. This suggests that panelist were able to find differences in color

between samples. In order to confirm if there were differences in color between sausages, a ranking test was also carried out. The results of this test confirmed there are differences between samples when assessing sausage overall darkness, but panelists seemed to do not associate their preference according to the color. As for this, overall acceptability was studied after 45 days of storage and consumers did not show differences in preference between treatments for any of the studied factors (Table 5). It should be taken into account that Catalan consumers, thanks to the big quantity of small- and large-scale producers, are used to a broad variability in this type of dry-cured sausages in their local markets. This fact suggests that the panelists have different preferences about color among them and/or appreciate other characteristics apart from the sausage color. This hypothesis is also in agreement with the consumer test which showed that the addition of CP at 2 doses (0.23% and 0.46%) had no influence on overall acceptability (Table 5). In cured cooked ham produced using the same vegetable extract, the addition of CP at 0.2% had no effect on sensory attributes whereas at 0.3% the panel described an increased vegetable aroma (11).

Apart from color, the addition of the tocopherol extract influences lipid oxidation since it is a good antioxidant. In consequence, the addition of the tocopherol extract reduced the oxidative status (LHPC and TBA values) of the sausages but also reduced all the induced FOX parameters with the exception of TMAX that remains unaffected (Tables 5 and 6). Lipid oxidation is increased with storage time thus the addition of this extract in the formulation can be a useful strategy to prevent lipid oxidation in dry-fermented sausages. The LHPC and the induced FOX parameters are highly correlated between them and, in addition, LHPC, Final LHP, MAXLHP and AUC are highly correlated with the tocopherol analogs amounts found in sausages after 0 (data not shown) and after 45 days of storage (Table 7) thus

548 supporting that these parameters, with the exception of TMAX, can be good markers of lipid
549 oxidation.
550 Increased amounts in all four tocol analogs were obtained by the addition of *S. carnosus*
551 (Table 4). This phenomenon might be explained by the rapid reduction of nitrate to nitrite
552 thanks to their nitrate reductase activity which would favor a higher protection against
553 oxidation at the beginning of the curing process. Studying the addition of nitrite instead of
554 nitrate, Walsh et al. (37) found that the dose of 100 mg of nitrite / kg meat in cured pork
555 products reduced TBA values in comparison to those products that only received 50 mg/kg.
556 The significant interactions involving starter culture and nitrate source should be considered
557 since *S. carnosus* was able to reduce all nitrate when low doses were applied thus provoking
558 no more nitrite supply during long term ripening and/or storage. Likely, the storage under the
559 modified atmosphere at 4 C did not allow a rapid progression of the oxidation since the
560 recorded TBA values were low and showed no differences between the two types of culture.
561 However, carrying out the induced FOX method, a significantly higher MAXLHP, Final LHP
562 and AUC values were recorded when *S. carnosus* had been added (Table 6) which is likely
563 indicating the protective role of residual nitrite against oxidation. In addition, LHPC, TBA
564 and induced FOX oxidation parameters increased after 45 days of storage which indicates
565 that stored sausages underwent slight oxidation under these conditions (Tables 5 and 6). This
566 could be due to the loss of tocol analogs (Table 4) and other possible reductants present in the
567 sample such as ascorbate during storage.
568 Collectively, these results indicate that organic sausages can be produced without affecting
569 significantly the quality and consumer acceptability of the product. Moreover, the addition of
570 *S. carnosus* is useful in reducing the residual levels of nitrate without any effect on TBA

571 values after 45 days of storage under an atmosphere containing 20% CO_2 plus 80% N_2 at
572 refrigeration. However, according to the induced-FOX values the combination of low doses
573 of nitrate with addition of *S. carnosus* may lead to an increased susceptibility to oxidation.
574 The substitution of chemical grade nitrate source for CP is a useful strategy for organic
575 production and at the lower dose it does not affect color formation or any other parameter in
576 comparison to the conventional procedures.
577
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587
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731 **Table 1**. Sausage formulation treatments

Treatments	Tocopherols (mg/kg)[a]	Starter culture[b]	Type of nitrate source and dose (mg/kg)[c]
1	0	Conventional	Celery conc. powder 70
2	0	Conventional	Celery conc. powder 140
3	0	Conventional	Chemical grade 70
4	0	Conventional	Chemical grade 140
5	0	*S. carnosus*	Celery conc. powder 70
6	0	*S. carnosus*	Celery conc. powder 140
7	0	*S. carnosus*	Chemical grade 70
8	0	*S. carnosus*	Chemical grade 140
9	200	Conventional	Celery conc. powder 70
10	200	Conventional	Celery conc. powder 140
11	200	Conventional	Chemical grade 70
12	200	Conventional	Chemical grade 140
13	200	*S. carnosus*	Celery conc. powder 70
14	200	*S. carnosus*	Celery conc. powder 140
15	200	*S. carnosus*	Chemical grade 70
16	200	*S. carnosus*	Chemical grade 140

732 [a] Expressed as sum average of tocopherol analogs. The tocopherol extract contains α-, β-, γ-
733 and δ-tocopherol analogs at the concentrations of 109 ± 4, 12.7 ± 0.3, 476 ± 12, and 189 ± 5
734 g/kg, respectively.

735 [b] Conventional starter culture includes *Lactobacillus sakei* and *Staphylococcus xylosus*. The
736 conventional starter culture was also added in the *Staphylococcus carnosus* treatments.
737 [c] Addition of chemically pure KNO_3 or celery concentrate powder providing different doses
738 of nitrate, 70 or 140 mg expressed as $NaNO_3$/kg
739
740

741 **Table 2**. Effect of sausage formulation factors and processing points (after inoculation in the

742 raw mix batter, after curing at 0 days and after 45 days storage) on microbial counts[a]

	Microbial counts (log CFU/g)	
	Lactobacilli[b]	**Staphylococci[c]**
Tocopherols[d]		
0	8.4	6.9
200	8.2	7.0
SEM[e]	0.085	0.082
Starter culture[f]		
Conventional	8.2	6.9
S. carnosus	8.4	7.1
SEM	0.085	0082
Nitrate source and dose[g]		
Chemical 70	8.3	7.0
Chemical 140	8.3	7.1
Celery 70	8.2	6.9
Celery 140	8.3	6.9
SEM	0.12	0.12
Time		
raw mix batter	8.2 x	7.5 y
0 days	8.6 y	7.2 y
45 days	8.1 x	6.2 x
SEM	0.10	0.10

743 [a] Values given in this table correspond to least-squares means obtained from multifactor ANOVA (n = 48).

744 Least-squares means within the same column with different letters differ significantly ($P \leq 0.05$).

745 [b] Microbial counts expressed as the logarithm of lactobacilli colony-forming units per g of dried sample.
746 Significant interactions between tocopherols x storage time for lactobacilli ($P = 0.005$) were found.
747 [c] Microbial counts expressed as the logarithm of staphylococci colony-forming units per g of dried sample A
748 significant interaction between starter culture x nitrate source ($P = 0.001$) for staphylococci was found.
749 [d] Tocopherol dose expressed in mg of tocopherol analogs/kg meat.
750 [e] SEM means standard error of the mean.
751 [f] Conventional starter culture includes *Lactobacillus sakei* and *Staphylococcus xylosus*. The *Staphylococcus*
752 *carnosus* starter culture also includes the conventional starter culture.
753 [g] Type of source and dose of nitrate expressed in mg $NaNO_3$/kg meat.
754
755

Table 3. Effect of formulation factors and storage time on sausage moisture, residual nitrate, residual nitrite and CIE L* a* b* color values[a]

	moisture (%)[b]	**Residual nitrate** (mg/kg)[c]	**Residual nitrite** (mg/kg)[d]	**L*** [e]	**a*** [f]	**b*** [g]
Tocopherols[h]						
0	25.3 x	27	0.36	37.52 x	15.38 x	8.32 x
200	26.0 y	26	0.33	38.36 y	16.21 y	8.61 y
SEM[i]	0.19	1.5	0.012	0.063	0.054	0.019
Starter culture[j]						
Conventional	25.8	53 y	0.35	37.85 x	15.71 x	8.43 x
S. carnosus	25.6	Tr x	0.34	38.00 y	15.88 y	8.50 y
SEM	0.19	1.5	0.012	0.063	0.054	0.019
Nitrate source and dose[k]						
Chemical 70	25.6	9 x	0.36	37.18 x	15.78 x	8.36 x
Chemical 140	25.3	48 y	0.35	37.85 x	15.77 x	8.43 x
Celery 70	25.7	Tr[l] x	0.34	37.82 x	15.70 x	8.43 x
Celery 140	26.0	49 y	0.33	38.34 y	15.93 x	8.66 y
SEM	0.27	2.1	0.017	0.091	0.077	0.027
Storage time						
0 days	25.8	25	0.38 y	38.17 y	16.30 y	8.52 y
45 days	25.6	28	0.31 x	37.71 x	15.29 x	8.42 x
SEM	0.19	1.5	0.012	0.063	0.054	0.019

[a] Values given in this table correspond to least-squares means obtained from multifactor ANOVA (n = 32, 32 and 135 for moisture, nitrate and nitrite analyses, and color measurements, respectively). Least-squares means within the same column for the same factor with different letters differ significantly ($P \leq 0.05$).

[b] Significant interactions between nitrate source x starter culture ($P = 0.005$) and between nitrate source x tocopherols ($P = 0.007$) for moisture were found.

[c] Residual nitrate is expressed as mg of $NaNO_3$ per kg of sausage in dry weight basis. A significant interaction between starter culture x nitrate source ($P \leq 0.001$) for residual nitrate was found.

[d] Residual nitrite is expressed as mg of $NaNO_2$ per kg of sausage in dry weight basis A significant interaction between nitrate source x storage time ($P = 0.0042$) for residual nitrite was found.

[e] Significant interactions between starter culture x nitrate source ($P \leq 0.001$), starter culture x storage time ($P = 0.002$), nitrate source x tocopherols ($P \leq 0.001$) and nitrate source and storage time ($P \leq 0.001$) for L* values were found.

[f] Significant interactions between starter culture x tocopherols ($P = 0.003$), starter culture x nitrate source ($P \leq 0.001$), starter culture x storage time ($P = 0.014$), nitrate source x tocopherols ($P \leq 0.001$) and nitrate source x storage time ($P = 0.001$) for a* values were found.

[g] Significant interactions between starter culture x tocopherols ($P = 0.002$), starter culture x nitrate source ($P \leq 0.001$), nitrate source x tocopherols ($P \leq 0.001$) and nitrate source x storage time ($P \leq 0.001$) for b* values were found.

[h] Tocopherol dose expressed in mg of tocopherol analogs/kg meat.

[i] SEM means standard error of the mean.

[j] Conventional starter culture include *Lactobacillus sakei* and *Staphylococcus xylosus*, the *Staphylococcus carnosus* starter culture also includes the conventional starter culture.

[k] Type of source and dose of nitrate expressed in mg $NaNO_3$/kg meat.

[l] Tr means traces. The analyte amounts were found between the limits of detection and quantification of the method used.

Table 4. Effect of formulation factors and storage time on sausage tocol analogs[a]

	α-tocopherol (mg/kg)[b]	β-tocopherol (mg/kg)[c]	γ-tocopherol (mg/kg)[d]	δ-tocopherol (mg/kg)[e]	α-tocotrienol (m/kg)[f]
Tocopherols[g]					
0	18.8 x	0.3 x	1 x	Tr[h] x	0.77 x
200	74.5 y	7.3 y	260 y	47.3 y	1.22 y
SEM[i]	0.91	0.08	3.0	0.38	0.04
Starter culture[j]					
Conventional	45.0 x	3.7 x	126 x	23.0 x	0.96
S. carnosus	48.2 y	3.9 y	136 y	24.3 y	1.04
SEM	0.91	0.08	3.0	0.38	0.04
Nitrate source and dose[k]					
Chemical 70	43.3 x	3.5 x	123	22.4	0.83 x
Chemical 140	47.4 xy	3.9 xy	132	24.0	1.10 y
Celery 70	47.2 xy	3.8 xy	130	23.7	1.00 xy
Celery 140	48.7 y	4.0 x	139	24.6	1.06 xy
SEM	1.3	0.11	4.3	0.54	0.06
Storage time					
0 days	52.6 y	4.1 y	140 y	24.8 y	1.16 x
45 days	40.7 x	3.5 x	122 x	22.5 x	0.83 x
SEM	0.91	0.08	3.0	0.38	0.04

[a] Values given in this table correspond to least-squares means obtained from multifactor ANOVA (n = 64). Least-squares means within the same column for the same factor with different letters differ significantly ($P \leq 0.05$). β-, γ- and δ-tocotrienols were normally below the quantification limits and, in consequence, were not reported.

[b] Results are expressed as mg α-tocopherol per kg of sausage in dry weight basis. Significant interactions between starter culture x nitrate source ($P = 0.030$), starter culture x storage time ($P = 0.023$) and storage time x tocopherols ($P = 0.015$) for α-tocopherol were found.

[c] Results are expressed as mg β-tocopherol per kg of sausage in dry weight basis. Significant interactions between starter culture x tocopherols ($P = 0.046$), starter culture x nitrate source ($P \leq 0.001$), tocopherols x nitrate source ($P = 0.045$) and tocopherols x storage time ($P \leq 0.001$) for β-tocopherol were found.

[d] Results are expressed as mg γ-tocopherol per kg of sausage in dry weight basis. Significant interactions between starter culture x nitrate source ($P = 0.021$), starter culture x tocopherols ($P = 0.031$), and storage time x tocopherols ($P \leq 0.001$) for γ-tocopherol were found.

[e] Results are expressed as mg δ-tocopherol per kg of sausage in dry weight basis. Significant interactions between starter culture x tocopherols ($P = 0.032$), starter culture x nitrate source ($P \leq 0.001$) and storage time x tocopherols ($P \leq 0.001$) for δ-tocopherol were found.

[f] Results are expressed as mg α-tocopherol per kg of sausage in dry weight basis. Significant interactions between starter culture x nitrate source ($P = 0.003$) and starter culture x storage time ($P = 0.043$) were found for α-tocotrienol.

[g] Tocopherol dose expressed in mg of tocopherol analogs/kg meat.

[h] Tr means traces. The analyte amounts were found between the limits of detection and quantification of the method used.

[i] SEM means standard error of the mean.

[j] Conventional starter culture include *Lactobacillus sakei* and *Staphylococcus xylosus*. The *Staphylococcus carnosus* starter culture also includes the conventional starter culture.

[k] Type of source and dose of nitrate expressed in mg $NaNO_3$/kg meat.

814 **TABLE 5.** Effect of formulation factors and storage time on sausage lipid hydroperoxide
815 content (LHPC), thiobarbituric acid (TBA) values and consumers' overall acceptability[a]

	LHPC (mmol CHP eq/kg)[b]	TBA (μg MDA/kg)[c]	Overall acceptability
Tocopherols[d]			
0	352 y	300 y	-0.3
200	73 x	30 x	0.1
SEM[e]	38	22	0.33
Starter culture[f]			
Conventional	203	170	-0.3
S. carnosus	222	170	0.1
SEM	38	22	0.33
Nitrate source and dose[g]			
Chemical 70	348 x	220	-0.5
Chemical 140	237 x	130	0.4
Celery 70	129 x	140	0.4
Celery 140	134 x	180	-0.7
SEM	54	32	0.46
Storage time			
0 days	138 x	90 x	N.A.[h]
45 days	287 y	240 y	
SEM	38	22	

816 [a] Values given in this table correspond to least-squares means obtained from multifactor ANOVA (n = 64, 64
817 and 192 for LHPC, TBA values and consumers' acceptability, respectively). Least-squares means within the
818 same column for the same factor with different letters differ significantly ($P \leq 0.05$).
819 [b] Results are expressed as mmol of cumene hydroperoxide equivalents per kg of sausage in dry weight basis.
820 Significant interactions between tocopherols x nitrate source ($P = 0.006$), tocopherols x storage time ($P = 0.007$)
821 and nitrate source x storage time ($P = 0.047$) for LHPC were found.

822 [c] Results are expressed as μg of malondialdehyde per kg of sausage in dry weight basis. Significant interactions
823 between storage time x tocopherols ($P \leq 0.001$), storage time x starter culture ($P = 0.013$) and starter culture x
824 nitrate source ($P = 0.002$) for TBA were found.
825 [d] Tocopherol dose expressed in mg of tocopherol analogs/kg meat.
826 [e] SEM means standard error of the mean.
827 [f] Conventional starter culture include *Lactobacillus sakei* and *Staphylococcus xylosus*. The *Staphylococcus*
828 *carnosus* starter culture also includes the conventional starter culture.
829 [g] Type of source and dose of nitrate expressed in mg $NaNO_3$/kg meat.
830 [h] N.A. means not analyzed at time 0 days.
831

TABLE 6. Effect of formulation factors and storage time on sausage maximum lipid hydroperoxide value (MAXLHP), time to reach the maximum lipid hydroperoxide value (TMAX), oxidation rate (OR), final lipid hydroperoxide value (Final LHP) and area under the curve (AUC)[a]

	MAXLHP (mmol CHP eq kg $^{-1}$)[b]	**TMAX** (h)[c]	**OR** (μmol CHP eq kg^{-1} h^{-1})[d]	**Final LHP** (mmol CHP eq kg $^{-1}$)[e]	**AUC** (mol CHP eq kg^{-1} h)[f]
Tocopherols[g]					
0	2900 y	48	86 y	2190 y	500 y
200	1500 x	54	26 x	980 x	251 x
SEM[h]	104	2.2	6.6	90	19
Starter culture[i]					
Conventional	2100 x	50	54	1390 x	343 x
S. carnosus	2400 y	53	59	1790 y	402 y
SEM	104	2.2	6.6	90	19
Nitrate source and dose[j]					
Chemical 70	2900 y	39 x	105 x	1900 y	478 y
Chemical 140	2000 x	54 y	51 y	1500 xy	344 x
Celery 70	1900 x	56 y	31 y	1400 x	312 x
Celery 140	2200 x	56 y	38 y	1600 xy	357 x
SEM	147	3.2	9.4	127	28
Storage time					
0 days	1700 x	44 x	39 x	1120 x	296 x
45 days	2700 y	59 y	74 y	2050 y	450 y
SEM	104	2.2	6.6	90	19

[a] Values given in this table correspond to least-squares means obtained from multifactor ANOVA (n = 64). Least-squares means within the same column for the same factor with different letters differ significantly ($P \leq 0.05$).

[b] Results are expressed as mmol of cumene hydroperoxide equivalents per kg of sausage in dry weight basis. Significant interactions between tocopherols x starter culture (P = 0.040), tocopherols x nitrate source (P = 0.005), tocopherols x storage time ($P \leq 0.001$), starter culture x nitrate source (P = 0.004), starter culture x storage time (P = 0.006) and storage time x nitrate source (P = 0.007) for MAXLHP were found.

[c] Significant interactions between nitrate source x tocopherols (P = 0.001), nitrate source x storage time (P = 0.015) and storage time x tocopherols (P = 0.017) for TMAX were found.

[d] Results are expressed as μmol of cumene hydroperoxide equivalents per kg of sausage in dry weight basis and h. Significant interactions between nitrate source x tocopherols ($P \leq 0.001$), nitrate source x starter culture (P = 0.008), nitrate source x storage time (P =0.007) and storage time x tocopherols ($P \leq 0.001$) for OR were found.

[e] Results are expressed as mmol of cumene hydroperoxide equivalents per kg of sausage in dry weight basis. Significant interactions between starter culture x tocopherols ($P \leq 0.001$), starter culture x nitrate source (P = 0.030) and starter culture x storage time (P = 0.001) for Final LHP were found.

[f] Results are expressed as mol of cumene hydroperoxide equivalents and h per kg of sausage in dry weight basis. Significant interactions between tocopherols x nitrate source (P = 0.009), tocopherols x starter culture (P = 0.039), tocopherols x storage time ($P \leq 0.001$), starter culture x nitrate source (P = 0.009) and starter culture x storage time (P = 0.009) for AUC were found.

[g] Tocopherol dose expressed in mg of tocopherol analogs/kg meat.

[h] SEM means standard error of the mean.

[i] Conventional starter culture include *Lactobacillus sakei* and *Staphylococcus xylosus*. The *Staphylococcus carnosus* starter culture also includes the conventional starter culture.

[j] Type of source and dose of nitrate expressed in mg $NaNO_3$/kg meat.

Table 7. Spearman correlation coefficients between lipid hydroperoxide content, induced FOX parameters and tocol content in sausages, after storage for 45 days[a]

	LHPC	MAXLHP	TMAX	OR	Final LHP	AUC	α-T	β-T	γ-T	δ-T	α-T3
	1[b]	0.75	-0.43	0.73	0.78	0.77	-0.77	-0.75	-0.78	-0.81	-0.69
LHPC	.	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	16	16	16	16	16	16	16	16	16	16	16
		1.00	-0.37	0.97	0.98	0.97	-0.79	-0.74	-0.78	-0.69	-0.83
MAXLHP		.	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		16	16	16	16	16	16	16	16	16	16
			1.00	-0.50	-0.42	-0.45	0.45	0.14	0.41	0.33	0.49
TMAX			.	0.05	0.10	0.08	0.08	0.61	0.11	0.21	0.05
			16	16	16	16	16	16	16	16	16
				1.00	0.97	0.97	-0.82	-0.75	-0.82	-0.73	-0.86
OR				.	0.00	0.00	0.00	0.00	0.00	0.00	0.00
				16	16	16	16	16	16	16	16
					1.00	1.00	-0.80	-0.78	-0.84	-0.72	-0.83
Final LHP					.	0.00	0.00	0.00	0.00	0.00	0.00
					16	16	16	16	16	16	16
						1.00	-0.80	-0.77	-0.84	-0.72	-0.84
AUC						.	0.00	0.00	0.00	0.00	0.00
						16	16	16	16	16	16
							1.00	0.87	0.91	0.90	0.84
α-T							.	0.00	0.00	0.00	0.00
							16	16	16	16	16
								1.00	0.95	0.93	0.78
β-T								.	0.00	0.00	0.00
								16	16	16	16
									1.00	0.93	0.86
γ-T									.	0.00	0.00
									16	16	16
										1.00	0.82
δ-T										.	0.00
										16	16

α-T3		1.00 . 16

[a] LHPC = lipid hydroperoxide content, MAXLHP = maximum lipid hydroperoxide value, TMAX = time the maximum lipid hydroperoxide value was achieved, OR = oxidation rate, Final LHP = the final lipid hydroperoxide value, AUC = are under the curve, α-T = α-tocopherol, β-T = β-tocopherol, γ-T = γ-tocopherol, δ-T = δ-tocopherol and α-T3 = α-tocotrienol.

[b] Spearman correlation coefficient, P value and number of samples are stated respectively one below the other.