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## Peptide from RuBisCO protein hydrolysate and its application in beef meat preservation

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1 **Peptide from RuBisCO protein hydrolysate and its application in beef meat**  
2 **preservation**

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25 **Abstract** – The objective of this work was to purify a small (705 Da) and hydrophilic  
26 antimicrobial peptide (Arg-Asp-Arg-Phe-Leu) from RuBisCO protein hydrolysate and to  
27 evaluate its effect on the microbiological and oxidative stability of minced beef meat during  
28 refrigeration. The TBARS values were significantly affected by the storage period and the  
29 concentration of bioactive peptide. The antioxidant effect was similar to that of the BHT used  
30 as control. The counts of all determined microbiological indicators were significantly affected  
31 by the addition of the Arg-Asp-Arg-Phe-Leu. Arg-Asp-Arg-Phe-Leu can be effectively  
32 utilized as food ingredients in meat for enhancing their shelf life and oxidative stability.

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34 **Index Terms:** Antioxidant; antimicrobial; bioactive peptide, beef meat.

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## Introduction

50  
51 Fresh products as minced meat contains adequate amount of water and abundance of  
52 proteins and essential nutrients with favorable pH for supporting microbial growth. Meat is  
53 highly perishable products and requires appropriate preservation (Domínguez et al. 2018).  
54 Therefore, several synthetic additives have long been directly applied as food additives or  
55 used as processing aids with primary intention of prolonging shelf life and preserving quality  
56 of meat and meat products (Munekata et al., 2020). The application of chemical preservatives  
57 such as butylated hydroxytoluene (BHT) and propyl gallate (PG) has been questioned because  
58 of the potential toxic and carcinogenic effects (Hashemi et al. 2017). Not only pressure of the  
59 consumer health concerns, but also a trend towards natural food additives so called “clean-  
60 labeling” has driven exploring of natural antimicrobial compounds as an alternative to  
61 synthetic food additives (Domínguez et al. 2019; Lorenzo et al. 2018). Advancement in  
62 bioactive peptide research has showed that the peptides derived from food proteins could be  
63 natural potential candidates to replace the synthetic compounds (Chakrabarti et al., 2018;  
64 Wang et al., 2015). Indeed, antimicrobial peptides of industrial waste could be used as  
65 preservatives (Lafarga & Hayes, 2014). To date, several animal peptides such as lysozyme,  
66 lactoferrin, and nisin are approved for application in meat and meat products (Silveira et al.,  
67 2021).

68 Recently, the food industries have taken interest to peptides derived from agro-  
69 industrial waste. Green juice derived from bio-refinery of Alfalfa (*Medicago sativa*), is often  
70 considered as a source of pollution, but it is an excellent protein-rich source and has become  
71 an alternative for peptides generation with great interest for food formulation (Hadidi et al.,  
72 2019). Ribulose-1,5-bisphosphate carboxylase oxygenase, most commonly known by the  
73 shorter name RuBisCO. It's the most abundant plant protein and possesses unique  
74 characteristics, a favorable nutritional profile, beneficial bioactivities, and desirable physical

75 attributes, making it a promising candidate for use in food applications (Grácio et al., 2023).  
76 Several studies showed that RuBisCO have been reported to contain several bioactive  
77 peptides compared to commonly consumed food proteins, except milk proteins (Corrêa et al.,  
78 2023). In recent previous studies antibacterial and antioxidant peptides derived from the  
79 enzymatic hydrolysis of Alfalfa RuBisCO were isolated and identified (Kobbi et al., 2015;  
80 Kobbi et al., 2017). The antibacterial peptide (Arg-Asp-Arg-Phe-Leu) was previously  
81 identified from protein hydrolysates of Alfalfa RuBisCO prepared with pepsin® (Kobbi et al.,  
82 2015). Arg-Asp-Arg-Phe-Leu a small (705 Da) peptide with one positive charges at pH 7  
83 belongs to the great subunit of the RuBisCO, which has a large antibacterial spectrum as  
84 against Gram positive and Gram-negative bacteria (Kobbi et al., 2015). This bioactive peptide  
85 has several attractive properties and attributes that make it a potential promising candidate for  
86 application as preservative in the storage and distribution of various meat products. The  
87 objective of this work was to purify a small (705 Da) and hydrophilic antimicrobial peptide  
88 (Arg-Asp-Arg-Phe-Leu) from RuBisCO protein hydrolysate and to evaluate its effect on the  
89 microbiological and oxidative stability of minced beef meat during refrigeration.

### 90 **Materials and methods**

91 All common chemicals and solvents were of analytical grade from commercial  
92 sources: Sigma-Aldrich (Saint-Quentin Fallavier, France) or Flandres Chimie (Villeneuve  
93 d'Ascq, France). Synthetic peptide was obtained from GeneCust Europe (Biotechnology  
94 Laboratory of Luxembourg S.A). The beef meat was obtained from a local purveyor.

95 The purification of RuBisCO was realized by a simple method based on pH and  
96 solvent effect, according to the method described by Kobbi et al., (2017). Primary purification  
97 of an interest protein from a crude medium is specifically to eliminate impurities depending  
98 on the solubility and chemical properties of RuBisCO, as its overall charge, solubility or pHi.  
99 In order to isolate a soluble fraction, 100 g of green juice was solubilized in 1 L of distilled

100 water or 1 L of ammonia (NH<sub>4</sub>OH) at 0.1 M and pH 11. Suspensions were centrifuged at  
101 10,000 g for 20 min at 4°C to eliminate the green protein and all debris. The RuBisCO  
102 containing base 1 at pH 10 was repurified to retrieve the second supernatant 2. Base 2 was  
103 repurified again to recover supernatant 3. After three successive centrifugations at pH 10, all  
104 insoluble fractions were removed, and, hence, three supernatants were summed up to recover  
105 all RuBisCO. Proteins were then precipitated by the acidification of the medium at pH 3 using  
106 10 mM of formic acid. This was followed by a last centrifugation step at 10,000 g at 4 °C for  
107 20 min. The precipitate was washed by ultrapure water and RuBisCO was then lyophilized.  
108 A 10% (w/v) aqueous solution of the lyophilized RuBisCO was adjusted to pH 10 and then  
109 centrifuged at 10,000 g for 5 min. The supernatant was adjusted to optimal pH (pH 3.0) and  
110 temperature (37°C) for pepsin<sup>®</sup>. The hydrolysis reaction was started by the addition of the  
111 enzyme at a 1:100 (U/mg) enzyme/ protein ratio. The enzymatic reaction was conducted at 37  
112 °C and pH 3 for 24 h. The hydrolysis reactions are stopped after 24 h by increasing the pH to  
113 10 with 1 M NaOH and then stored at -20 °C for further use. The proportion of none  
114 hydrolyzed RuBisCO and then hydrolyzed RuBisCO was deduced from the peak areas by  
115 HPLC.

116 The degree of hydrolysis (DH) is defined as the ratio of the number of peptide bonds cleaved  
117 to the total number of peptide bonds of protein substrate. DH is determinate by the OPA  
118 (o-phthaldialdehyde) method using leucine as standard (Nielsen et al., 2001). A fresh OPA  
119 solution was prepared extemporaneously with 25 mL of 100 mM sodium tetrahydroborate, 5  
120 mL of 10% SDS solution (w/v), 80 mg of OPA (dissolved in 1 mL of methanol) and 0.01 mL  
121 of β-mercaptoethanol were adjusted to a final volume of 50 ml with distilled water. To assay  
122 proteolysis with RuBisCO protein as substrates, an aliquot (usually 0.01–0.05 ml containing  
123 5–100 mg proteins) was added to 1.0 ml of OPA reagent in a 1.5 ml quartz cuvette. The

124 solutions were mixed briefly and incubated for 2 min at room temperature, and then the  
125 absorbance at 340 nm was measured.

126 The RuBisCO hydrolysis was analyzed by RP-HPLC on a C4 column according to the  
127 process previously described with slight modifications ([Trovalset et al., 2007](#)). The liquid  
128 chromatographic system consisted of a Waters 600E automated gradient controller pump  
129 module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array  
130 detector. Spectral and chromatographic data were stored on a NEC Image 466 Image 466  
131 computer. Millennium software was used to plot, acquire, and analyze chromatographic data.  
132 Chromatographic processes were performed with a VydacC4 column (250 mm×10 mm). The  
133 mobile phase composed of water/trifluoroacetic acid (1000:1, v/v) was used as eluent A,  
134 while acetonitrile/trifluoroacetic acid (1000:1, v/v) was used as eluent B. The flow rate was  
135 0,6 ml/min. Samples were filtered through 0.22mm filters and then injected. The gradient  
136 applied was 0-10 min, 100% A; 25-95min, 21- 81% B; 96-110min, 100- 0% B; and the HPLC  
137 system were equilibrated for 10 min with 100% A. On-line UV absorbance scans were  
138 performed between 215 and 325 nm at a rate of one spectrum per second with a resolution of  
139 1.2 nm. Chromatographic analyzes were completed with Millennium software.

140 The RuBisCO hydrolysate was adjusted to pH 7.5 and NaCl was added to a fixed volume to  
141 reach a final concentration of 2.0 M of salt ([Adoui et al., 2013](#)). The solution was stirred for  
142 30 min at room temperature before being centrifuged at 10,000 g for 10 min at 4°C. The pellet  
143 was washed twice by centrifugation with phosphate buffer (5 mM, pH 7.5) to remove soluble  
144 proteins, and then resuspended in phosphate buffer (5 mM, pH 7.5, 100 mM NaCl) to 20  
145 times the original hydrolysate volume. The final pH of the peptide hydrolysate was adjusted  
146 between 1.5 to 2. After one hour stirring at 7 °C, the pellet was centrifuged (10,000 g, 10  
147 min), and the supernatant was recovered. The solution was stored at -20°C until performing  
148 the antibacterial assay and analysis by RP-HPLC.

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149 The molecular mass and peptide sequencing were done in positive ion mode using  
150 Electrospray ionisation-mass spectrometry (ESI-MS) and the tandem mass spectrometry  
151 (MS/MS), respectively. ESI mass spectrometry was performed using a triple quadrupole  
152 instrument Applied Biosystems API 3000 (PE Sciex, Toronto, Canada) equipped with an  
153 electrospray ion source. The system is controlled by the Analyst Software 1.4, allowing the  
154 control of the spectrometer, the analysis and the processing data. Interpretations of spectra  
155 MS-MS were made with the Bioanalyst software. The freeze-dried samples from RP-HPLC  
156 were dissolved in acetonitrile/water (20/80; v/v) containing 0.1% formic acid for the positive  
157 mode. The solution was injected (nebulised) uninterrupted, by a pump (Model 22, Harvard  
158 Apparatus, South Natick, USA) with a flow rate of 5  $\mu$ l/min. The potential of ionisation was  
159 of 5000 V in positive mode. At the time of the recording of the spectrum, 30 scans on average  
160 were added (MCA mode) for each spectrum. The gases used (nitrogen and air) were pure (up  
161 to 99%) and produced by a compressor Jun-Air 4000-40M and a nitrogen generator Whatman  
162 model 75-72 (Whatman Inc, Haverhill, MA the USA). The polypropylene glycol (PPG) was  
163 used for the calibration and the optimisation of the machine. The peptide sequence was  
164 determined from the CID spectrum of the protonated analyse  $[M+H]^+$  by MS/MS  
165 experiments. Peptide sequences were done using the bioanalyst software (Applied  
166 Biosystems, USA).

167 The antimicrobial peptide (Arg-Asp-Arg-Phe-Leu) was chemically synthesized by the  
168 conventional method: Fmoc solid phase synthesis, according to the method described by  
169 [Fields and Noble \(1990\)](#). Arg-Asp-Arg-Phe-Leu peptide was synthesized on a Fmoc-Arg  
170 (Pbf)-WangTentagel® S resin (0.25 mmol/g from Iris Biotech, 50  $\mu$ mol scale) using an  
171 Intavis peptide synthesizer (Proteigene) and standard Fmoc/tert-butyl. Activation of the amino  
172 acids (10 eq) was performed separately prior to the coupling step using a mixture of  
173 TBTU/HOBt/DIEA: 10 eq/10 eq/30 eq in DMF. A capping step was performed after each

174 coupling with a mixture of Ac<sub>2</sub>O/NMM/DMF: 5/10/75 v/v/v. At the end of the synthesis, the  
175 Fmoc protecting group was removed using 20% piperidine in DMF. The resin was washed  
176 with DMF (4 × 2 min) and CH<sub>2</sub>Cl<sub>2</sub> (4 × 2 min) and dried in vacuo. Deprotection and  
177 cleavage of the peptide from the resin was performed with a mixture of TFA/TIS/H<sub>2</sub>O: at  
178 room temperature. The mixture was precipitated in cold diethyl ether. The peptide was  
179 collected by centrifugation, dissolved in deionized water and lyophilized. Arg-Asp-Arg-Phe-  
180 Leu was purified by RP-HPLC.

181 The hemolytic activity of the Arg-Asp-Arg-Phe-Leu peptide was determined by methods of  
182 [Dathe et al. \(1996\)](#), slightly modified. Five milliliters of bovine blood were centrifuged at  
183 3500 rpm for 10 min to isolate erythrocytes, which were then washed three times with 10mM  
184 sodium phosphate, pH 7.5, containing NaCl 9 g/L (NaCl/Pi). The cell concentration stock  
185 suspension was adjusted to 10<sup>9</sup> cells /mL. The cell suspension (12 mL) along with varying  
186 amounts of peptide stock solution and the buffer were pipetted into Eppendorf tubes to give a  
187 final volume of 50 mL. The Eppendorf tubes with 2.5 × 10<sup>8</sup> cells/mL were then incubated at 37  
188 °C during 40 min. After centrifugation (5000 rpm, 5 min), 30 mL of supernatant was diluted  
189 in 500 mL water. The absorbance of the diluted solution was measured at 420 nm. The  
190 absorbance obtained after treating erythrocytes with only NaCl/Pi and SDS (0.2%) was taken  
191 as 0 and 100%, respectively. The test was carried out in triplicate.

192 The beef patties were composed with commercial beef containing 0.28% (w/w) of salt, 14%  
193 (w/w) of fat and about 18% of proteins. Salt (70% NaCl and 30% KCl) was added at a rate of  
194 1.5% to the minced meat. The sample was subdivided into four treatments: a negative control,  
195 the meat without additive, a positive control, the meat with two different BHT concentrations  
196 (0.1 and 0.5% (w/w)) and the meat with two different Arg-Asp-Arg-Phe-Leu peptide  
197 concentrations (0.1 % and 0.5% (w/w)). The meat sample preparation was realized under  
198 strong aseptic conditions to avoid contaminations. Patties of 10 g were placed in plastic foam

199 meat trays, wrapped with film polyethylene and kept at 4 °C for fourteen days. Four samples  
200 were taken from each treatment at fixed day in order to evaluate their oxidation and the  
201 potential microbial contamination. A strict sanitation procedure was followed during the  
202 preparation of meat samples to avoid microbial contamination.

203 Lipid oxidation was evaluated using the thiobarbituric acid-reactive substance (TBARS) as  
204 previously described by [Witte et al., \(1970\)](#). For each prepared formula five grams of minced  
205 meat were added to 12.5 mL of solution containing trichloroacetic acid (20% w/v) and  
206 phosphoric acid (2 M). The mixture was then adjusted to 25 mL of ultrapure water, shaken  
207 and filtered using Buchner funnel through Whatman paper. After that, 5 mL of the filtrate was  
208 then transferred into a glass test tube and an equal volume of 2-thiobarbituric acid (5 mM)  
209 was added. The test tube was then well shaken and incubated at room temperature for 17 h.  
210 The absorbance of the mixture was measured at 520 nm and the results were expressed as  
211 malondialdehyde equivalents per meat kilogram (mg MDA/kg meat).

212 Microbial count was carried out at days 0, 1, 3, 6, 9, and 11 of storage at 4°C according to the  
213 following procedure. 45 mL sterilized peptone solution (0.1% w/v) was added to each five  
214 grams of beef patty in a sterile stomacher bag, then homogenized. Decimal dilutions up to 10<sup>5</sup>  
215 prepared from the initial concentration (100 mg/mL) and 100 µL of the appropriate dilutions  
216 were spread out on different selective media. Total viable counts were determined using plate  
217 count agar (PCA), potato dextrose agar (PDA) for yeasts and molds counts, Man, Rogosa and  
218 Sharpe (MRS) agar for lactic acid bacteria and violet, red bile lactose (VRBL) agar for  
219 coliform counts were used after incubation at 37 °C for 24h. Microbiological count was  
220 expressed as the log<sub>10</sub> of colony-forming units per gram of patty (log<sub>10</sub> CFU/g).

221 All data were submitted to Analysis of Variance (ANOVA) and differences between means  
222 were evaluated by Duncan's Multiple Range Test. The data were analyzed using the

223 Statistical Package for the Social Sciences (SPSS) version 10.0 (Chicago, Illinois, USA).  
224 Differences were considered significant at  $p < 0.05$ .

## 225 **Results and Discussion**

226 Figure 1 shows the RP-HPLC chromatogram from the peptic RuBisCO proteins purified from  
227 green juice of Alafalfa. Generated hydrolysate (DH = 18%) was mainly intermediate peptides  
228 (produced from the beginning of hydrolysis and disappearing gradually as hydrolysis  
229 advance) (Abou-Diab et al., 2021). This standard condition following hydrolysis process led  
230 to a complex hydrolysate containing many peptides, intermediate or final, with various  
231 characteristics (Kleekayai & FitzGerald, 2022). The chromatographic profile (Figure 2A)  
232 showed the peptide population with retention times between 10 and 50 min which  
233 corresponding to antimicrobial peptides. According to the mass spectrometry analyses (Figure  
234 2B), one antimicrobial pentapeptide was identified: Arg-Asp-Arg-Phe-Leu. The MIC of the  
235 purified peptide varied between  $2.17 \pm 0.1$  mM (for *Listeria innocua* and *Bacillus subtilis*)  
236 and  $2.83 \pm 0.2$  mM (for *Escherichia coli* and *Micrococcus luteus*). Peptides showed an  
237 outstanding antibacterial activity against both Gram positive and Gram-negative bacteria by  
238 damaging the cell membrane integrity (Bi et al., 2020; Tu et al., 2019; Medeiros et al., 2018).  
239 Arg-Asp-Arg-Phe-Leu peptide did not cause hemolysis. The hemolytic activity of the peptide  
240 against human erythrocytes was determined as an indication of its toxicity towards  
241 mammalian cells. The highest concentration tested corresponding to this concentration of  
242 peptide. No hemolysis was observed up to a peptide concentration corresponding to five times  
243 the MIC. The positively charged residues can significantly reduce the toxicity of the peptides  
244 towards the mammalian cells, so with the increasing positive charges, the hemolytic activity  
245 of the peptides reduced reasonably (Lohan et al., 2023; Sengkhui et al., 2023).  
246 Significant changes in TBARS occurred over the sampling period when Arg-Asp-Arg-Phe-  
247 Leu peptide was added to chilled minced beef meat. Analysis of variance of the TBARS

248 values was determined between the different samples. The result showed that the TBARS  
249 values increased throughout the eleven days experiment. This fact was clearly marked for the  
250 control sample (without additives) which highest TBARS values value than all tested  
251 conditions by the end of storage (It goes from value  $0.56 \pm 0.07$  mg MDA for day 1 to a value  
252 of  $2.31 \pm 0.18$  mg MDA for day 11). While the TBARS values of the meat supplemented with  
253 either 0.5% or 0.1% (w/w) Arg-Asp-Arg-Phe-Leu peptide, were significantly lower ( $p <$   
254  $0.05$ ) than the meat without additives, used as control. Moreover, the efficiency in ground  
255 meat preservation increased proportionally with peptide concentration. But this difference was  
256 clear from day 6 (Figure 3) because no significant effect between the four treated groups has  
257 been observed during the first three days of the experiment, but their TBARS values were  
258 significantly lower than the control (Figure 3). From day 6, the power antioxidant of Arg-  
259 Asp-Arg-Phe-Leu at 0.5% (w/w) was higher ( $0.83 \pm 0.04$  mg MDA/kg) than the other two  
260 treated groups (peptide at 0.1% and BHT at 0.1%) with an average value of  $1.75 \pm 0.31$  mg  
261 MDA and  $1.25 \pm 0.13$  mg MDA respectively. Thus, the peptide at 0.5% (w/v) can protect beef  
262 meat against lipid oxidation and extend their storage time. The TBARS values of the meat  
263 supplemented with either peptide 0.5% or BHT 0.5% (w/v), used as reference, have no  
264 significant difference and were similar after 9 days of storage at 4 °C of an average value  
265 TBARS of  $0.91 \pm 0.21$  mg MDA that is two times less than the control ( $2.145 \pm 0.36$  mg MDA)  
266 (Figure 3). Consequently, the BHT at 0.5% (w/v) and Arg-Asp-Arg-Phe-Leu at 0.5% (w/w)  
267 reduced the lipid oxidation on meat of 60% (Figure 3) compared to the control. This tendency  
268 continued until 11 days. At the end of the experiment, the BHT at 0.5 (w/w) and Arg-Asp-  
269 Arg-Phe-Leu 0.5% (w/w) had no significant difference to protect against the phenomenon of  
270 lipid oxidation with a TBARS-value about of 0.96 mg MDA/kg meat. The results of the  
271 present study show that adding the Arg-Asp-Arg-Phe-Leu' peptide derived from protein  
272 hydrolysates of Alfalfa RuBisCO at 0.5% (w/w) protects beef patties against lipid oxidation.

273 The work confirms the results obtained by [Kobbi et al. \(2017\)](#) which revealed that the Alfalfa  
274 RuBisCO hydrolysate (DH = 18.8 %) exhibited strong antioxidant abilities, including the  
275 prevention of linoleic acid oxidation, reduction of ferric ion, and stability of ABTS\*.  
276 Therefore, green juice derived from bio-refinery of Alfalfa offers a promising source of  
277 antioxidant peptides of interest for various functional food applications. The antioxidant  
278 activity of this peptide was probably due to its low molecular weight (705 Da) and the  
279 presence of aromatic residue Phenylalanine (Phe) which can quench free radicals by direct  
280 electron transfer to act as an antioxidant ([Geng et al., 2023](#); [Jeampakdee et al., 2020](#); [Chi et  
281 al., 2015](#)). Peptides with highly potent inhibitory activity have hydrophobic amino acids as  
282 leucine (Leu), at the N-terminal position in the sequences ([Shi et al., 2022](#); [Nwachukwu et al.,  
283 2019](#)). The microbial count increased proportionally with the storage time and reached the  
284 highest values at the end of chill period. Among the experimental groups, the negative control  
285 group showed the most rapid increase in the number of total microorganisms (from  $4.29 \pm$   
286  $0.04$  to  $8.14 \pm 0.31$  log CFU/g), followed by samples treated with Arg-Asp-Arg-Phe-Leu  
287 peptide and BHT ([Figure 4a](#)). Arg-Asp-Arg-Phe-Leu peptide efficiency on bacterial growth  
288 inhibition was tested using 0.1%, and 0.5 % concentrations. In general, the microbial growth  
289 decreased significantly with the addition of Arg-Asp-Arg-Phe-Leu peptide and with the  
290 highest concentrations of this compound during the storage period ([Figure 4a](#)). Nevertheless,  
291 the group with a 0.5% (w/w) of Arg-Asp-Arg-Phe-Leu peptide showed the slowest increase in  
292 the number of total viable colonies until the eleventh day ( $7.12 \pm 0.12$  log CFU/g). The  
293 coliform count on a VRBL medium shows that no significant difference ( $p > 0.05$ ) has been  
294 observed between the four treated groups with additives during the first three days ([Figure  
295 4b](#)). But beyond this day, the group with Arg-Asp-Arg-Phe-Leu peptide at 0.5% (w/w)  
296 showed efficiency more important and comparable to that of BHT 0.5% (w/w) with the  
297 coliform counts of  $4.73 \pm 0.04$  log CFU/g, at day 11. Therefore, this peptide can reduce by

298 32% the number of total coliforms in meat compared to the control. For the yeasts and molds,  
299 the most effective additives were the Arg-Asp-Arg-Phe-Leu at 0.5% (w/w) and BHT 0.5%  
300 (w/w), with the yeasts and molds counts of  $5.64 \pm 0.25$  and  $5.63 \pm 0.06$  log CFU/g,  
301 respectively, at day 11 (Figure 4c). These effects were similar to BHT 0.5% (w/w) (no  
302 significant difference). On the other hand, the groups treated with BHT 0.1% (w/w) and Arg-  
303 Asp-Arg-Phe-Leu at 0.1% (w/w) are less effective and they were presented no significant  
304 difference with the yeasts and molds counts  $6.28 \pm 0.25$  and  $6.19 \pm 0.25$  log CFU/g  
305 respectively, at day 11, compared to group without additive with  $7.11 \pm 0.62$  log CFU/g. At  
306 last, for the growth of lactic acid bacteria (Figure 4d), during the first three days no bacterial  
307 proliferation has been observed for the four treated groups with additives (from  $6.01 \pm 0.42$  to  
308  $6.35 \pm 0.12$  log CFU/g), except for the untreated group, the number of lactic acid bacteria was  
309 increased from  $2.23 \pm 0.81$  log CFU/g at day 0 to  $3.53 \pm 0.12$  log CFU/g at day 3. After 6  
310 days, three treated groups (with peptide 0.5%, BHT 0.1% and BHT 0.5%) showed a  
311 significant difference with the negative control group (Figure 4d). Once again, the most  
312 effective group were Arg-Asp-Arg-Phe-Leu at 0.5% (w/w) and BHT 0.5% (w/w). At this  
313 time, the microbial counts were of  $4.67 \pm 0.83$  log CFU/g for the untreated group and  $3.24 \pm$   
314  $0.18$  log CFU/g for the group treated with the Arg-Asp-Arg-Phe-Leu peptide at 0.5% (w/w).  
315 Many studies have reported the impact of antimicrobial peptides on meat and its products  
316 (Zagorec & Champomier-Verges, 2017). Antimicrobial peptide can potentially be used to  
317 extend the quality of highly perishable foods during storage (Gogliettino et al., 2020).  
318 Antimicrobial peptides have unique structural characteristics. The length of amino acids and  
319 the composition of antimicrobial peptides that have already been studied are extremely  
320 diverse. Their tendency to adapt to various structural changes when they come into contact  
321 with membranes is exclusive (Torres et al., 2019). The cationic peptides exert antibacterial  
322 activity by interacting with negatively charged bacterial membrane to increase membrane

323 permeability and lead to cell membrane lysis and cell content release. Upon approaching the  
324 cytoplasmic membrane through electrostatic interaction with the microbial membrane,  
325 antimicrobial peptides bind to the microbial membrane and interact with the anionic  
326 components of the plasma membrane (Rajagopal & Walker, 2017; Baek et al., 2016).

### 327 **Conclusion**

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- 329 1. A small antibacterial peptide from RuBisCO was isolated and purified.
- 330 2. Improvement of oxidative stability of minced beef meat during refrigeration.
- 331 3. The peptide reduced the lipid oxidation about 60% to delay meat rancidity.
- 332 4. Inhibition of the microbial growths under refrigeration during 14 days.
- 333 5. The peptide can be effectively utilized as food ingredients in meat products.

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### 341 **References**

- 342 ABOU-DIAB, M.; THIBODEAU, J.; FLISS, I.; DHULSTER, D.; NEDJAR, N.; BAZINET,  
343 L. Impact of conductivity on the performances of electro-acidification and enzymatic  
344 hydrolysis phases of bovine hemoglobin by electrodialysis with bipolar membranes for  
345 the production of bioactive peptides. **Separation and Purification Technology**, v.269,  
346 p.118650, 2021. DOI: <https://doi.org/10.1016/j.seppur.2021.118650>.
- 347 ADOUI, F.; BOUGHERA, F.; CHATAIGNE, G.; CHIHIB, N. E.; EL HAMEUR, H.;  
348 DHULSTER, P.; ZIDOUNE, M.N.; NEDJAR-ARROUME, N. A simple method to  
349 separate the antimicrobial peptides from complex peptic casein hydrolysate and

350 identification of novel antibacterial domains within the sequence of bovine  $\alpha$ s-casein.  
351 **International Review of Chemical Engineering**, v.5, p.179-187, 2013.

352 BAEK, M.H; KAMIYA, M.; KUSHIBIKI, T.; NAKAZUMI, T.; TOMISAW, A.S.; ABE, C.;  
353 KUMAKI, Y.; KIKUKAWA, T.; DEMURA, M.; KAWANO, K.; AIZAWA, T.  
354 Lipopolysaccharide-bound structure of the antimicrobial peptide cecropin P1  
355 determined by nuclear magnetic resonance spectroscopy. **Journal of Peptides Science**,  
356 v.22, p.214-21, 2016. DOI: <https://doi.org/10.1002/psc.2865>.

357 BI, J.; TIAN, C.; JIANG, J.; ZHANG, G.L.; HAO, H.; HOU H.M. Antibacterial Activity and  
358 Potential Application in Food Packaging of Peptides Derived from Turbot Viscera  
359 Hydrolysate. **Journal of Agricultural and Food Chemistry**, v.68, p.9968-9977, 2020.  
360 DOI: <https://doi.org/10.1021/acs.jafc.0c03146>.

361 CHAKRABARTI, S. ; GUHA, S. ; MAJUMDER, K. Food-derived bioactive peptides in  
362 human health: Challenges and opportunities. **Nutrients**, v.10, 1738. DOI :  
363 <https://doi.org/10.3390/nu10111738>.

364 CHI, C.F.; WANG, B.; WANG, Y.M.; ZHANG, B.; DENG, S.G. Isolation and  
365 characterization of three antioxidant peptides from protein hydrolysate of bluefin  
366 leatherjacket (*Navodon septentrionalis*) heads. **Journal of Functional Foods**, v.12, p.1-  
367 10, 2015. DOI: <https://doi.org/10.1016/j.jff.2014.10.027>.

368 CORREA, JAF.; DE MELO NAZARETH, T. ; ROCHA, G.F.D. ; LUCIANO, F.B. Bioactive  
369 Antimicrobial Peptides from Food Proteins: Perspectives and Challenges for  
370 Controlling Foodborne Pathogens. **Pathogens**, v. 12, 477, 2023. DOI :  
371 <https://doi.org/10.3390/pathogens12030477>.

372 DATHE, M.; SCHUMANN, M.; WIEPRECHT, T.; WINKLER, A.; BEYERMANN, M.;  
373 KRAUSE, E.; MATSUZAKI, K.; MURASE, O.; BIENERT, M. Peptide helicity and  
374 membrane surface charge modulate the balance of electrostatic and hydrophobic  
375 interactions with lipid bilayers and biological membranes. **Biochemistry**, v.35,  
376 p.12612-12620, 1996. DOI: <https://doi.org/10.1021/bi960835f>.

377 DOMINGUEZ, R.; PATEIRO, M.; GAGAOUA, M.; BARBA, F.J.; ZHANG, W.;  
378 LORENZO, J.M. A comprehensive review on lipid oxidation in meat and meat  
379 products. **Antioxidants**, v.8, 429, 2019. DOI: <https://doi.org/10.3390/antiox8100429>.

380 DOMÍNGUEZ, R.; BARBA, F.J.; GÓMEZ, B.; PUTNIK, P.; BURSAC KOVAČEVIĆ, D.;  
381 PATEIRO, M.; SANTOS, E.M.; LORENZO, J.M. Active packaging films with natural

382 antioxidants to be used in meat industry: A review. **Food Research International**,  
383 v.113, p.93–101, 2018. DOI: <https://doi.org/10.1016/j.foodres.2018.06.073>.

384 FIELDS, G.B.; NOBLE, R.L. Solid phase peptide synthesis utilizing  
385 9fluorenylmethoxycarbonyl amino acids. **International Journal of Peptide and**  
386 **Protein Research**, v.35, p.161-214, 1990. DOI: <https://doi.org/10.1111/j.1399-3011.1990.tb00939.x>.

388 GENG, M. ; FENG, X.; WU, X.; TAN, X.; LIU, Z.; LI, L.; HUANG, Y.; TENG, F.; LI, Y.  
389 Encapsulating vitamins C and E using food-grade soy protein isolate and pectin  
390 particles as carrier: Insights on the vitamin additive antioxidant effects. **Food**  
391 **Chemistry**, v.418, 135955, 2023. DOI:  
392 <https://doi.org/10.1016/j.foodchem.2023.135955>.

393 Gogliettino, M.; Balestrieri, M.; Ambrosio, R.L.; Anastasio, A.; Smaldone, G.; Proroga,  
394 Y.T.R.; Moretta, R.; Rea, I.; De Stefano, L.; Agrillo, B.; Palmieri, G. Extending the  
395 Shelf-Life of Meat and Dairy Products via PET-Modified Packaging Activated With the  
396 Antimicrobial Peptide MTP1. **Frontiers in Microbiology**, v.10, 2963, 2020. DOI:  
397 [10.3389/fmicb.2019.02963](https://doi.org/10.3389/fmicb.2019.02963).

398 GRACIO, M.; OLIVEIRA, S.; LIMA, A.; FERREIRA, R.B. RuBisCO as a protein source for  
399 potential food applications: A review, **Food Chemistry**, v.419, 135993, 2023. DOI:  
400 <https://doi.org/10.1016/j.foodchem.2023.135993>.

401 HADIDI, M.; IBARZ, A.; CONDE, J.; PAGAN, J. Optimisation of steam blanching on  
402 enzymatic activity, color and protein degradation of alfalfa (*Medicago sativa*) to  
403 improve some quality characteristics of its edible protein, **Food Chemistry**, v.276,  
404 p.591-598, 2019. DOI: <https://doi.org/10.1016/j.foodchem.2018.10.049>.

405 HASHEMI, S.M.B.; NIKMARAM, N.; ESTEGHLAL, S.; KHANEGHAH, A.M.;  
406 NIAKOUSARI, M.; BARBA, F. J.; ROOHINEJAD, S.; KOUBAA, M. (2017).  
407 Efficiency of ohmic assisted hydrodistillation for the extraction of essential oil from  
408 oregano (*Origanum vulgare* subsp. *viride*) spices. **Innovative Food Science &**  
409 **Emerging Technologies**, v.41, p.172–178, 2017. DOI:  
410 <https://doi.org/10.1016/j.ifset.2017.03.003>.

411 JEAMPAKDEE, P.; PUTHONG, S.; SRIMONGKOL, P.; SANGTANOO, P.; SAISAVOEY,  
412 T.; KARNCHANATAT, A. The apoptotic and free radical–scavenging abilities of the  
413 protein hydrolysate obtained from chicken feather meal. **Poultry Science**, v.99, p.1693-  
414 1704, 2020. DOI: <https://doi.org/10.1016/j.psj.2019.10.050>.

415 KLEEKAYAI, T.; FITZGERALD, R.J. Protein Hydrolysates and Peptides, Editor(s): Paul  
416 L.H. McSweeney, John P. McNamara, **Encyclopedia of Dairy Sciences** (Third  
417 Edition), Academic Press, p.54-166, 2022. DOI: [https://doi.org/10.1016/B978-0-12-](https://doi.org/10.1016/B978-0-12-818766-1.00183-5)  
418 [818766-1.00183-5](https://doi.org/10.1016/B978-0-12-818766-1.00183-5).

419 KOBBI, S.; BALTI, R.; BOUGATEF, A.; LE FLEM, G.; FIRDAOUS, L.; BIGAN, M.;  
420 CHATAIGNE, G.; CHAABOUNI, S.; DHULSTER, P.; NEDJAR, N. Antibacterial  
421 activity of novel peptides isolated from protein hydrolysates of RuBisCO purified from  
422 green juice alfalfa. **Journal of Functional Foods**, v.18, p.703-713, 2015. DOI:  
423 <https://doi.org/10.1016/j.jff.2015.09.007>.

424 KOBBI, S.; BOUGATEF, A.; LE FLEM, G.; BALTI, R.; MICKAEL, C.; FERTIN, B.;  
425 CHAABOUNI, S.; DHULSTER, P.; NEDJAR, N. Purification and Recovery of  
426 RuBisCO Protein from Alfalfa Green Juice: Antioxidative Properties of Generated  
427 Protein Hydrolysate. **Waste and Biomass Valorization**, v.8, p.493–504, 2017. DOI:  
428 <https://doi.org/10.1007/s12649-016-9589-y>.

429 LAFARGA, T.; HAYES, M. Bioactive peptides from meat muscle and byproducts:  
430 Generation, functionality and application as functional ingredients. **Meat Science**, v.98,  
431 p.227–239, 2014. DOI: <https://doi.org/10.1016/j.meatsci.2014.05.036>.

432 LOHAN, S.; KONSHINA, AG.; EFREMOV, RG.; MASLENNIKOV, I.; PARANG, K.  
433 Structure-Based Rational Design of Small  $\alpha$ -Helical Peptides with Broad-Spectrum  
434 Activity against Multidrug-Resistant Pathogens. **Journal of Medicinal Chemistry**,  
435 v.66, p.855-874, 2023. DOI: <https://doi.org/10.1021/acs.jmedchem.2c01708>.

436 LORENZO, J.M.; PATEIRO, M.; DOMINGUEZ, R.; BARBA, F.J.; PUTNIK, P.;  
437 KOVACEVIC, D.B.; SHPIGELMAN, A.; GRANATO, D.; FRANCO, F. Berries  
438 extracts as natural antioxidants in meat products: A review. **Food Research**  
439 **International**, v.106, p.1095–1104, 2018. DOI:  
440 <https://doi.org/10.1016/j.foodres.2017.12.005>.

441 JOHNSON, J.C.P.S.; RITA, C.S.S.; CAIO, G.O.; ALLAN R.F.M.; VICTOR, G.L.S.; EBER,  
442 A.A.M.; PAULA J.P.E. ; ANA, C.S.P.; JANE, S.R.C.; NILDA, F.F.S. Nisin and other  
443 antimicrobial peptides: Production, mechanisms of action, and application in active food  
444 packaging. **Innovative Food Science and Emerging Technologies**, v.48, p.179-194,  
445 2018. DOI: <https://doi.org/10.1016/j.ifset.2018.06.008>.

446 MUNEKATA, P.E.S.; ROCCHETTI, G.; PATEIRO, M.; LUCINI, L.; DOMINGUEZ, R.;  
447 LORENZO, J.M. Addition of plant extracts to meat and meat products to extend shelf-

448 life and health-promoting attributes: An overview. **Current Opinion in Food Science**,  
449 v.31, p.81–87, 2020. DOI: <https://doi.org/10.1016/j.cofs.2020.03.003>.

450 NIELSEN, P.M.; PETERSEN, D.; DAMBMANN, C. Improved method for determining food  
451 protein degree of hydrolysis. **Journal of Food Science**, v.66, p.642–646, 2001. DOI:  
452 <https://doi.org/10.1111/j.1365-2621.2001.tb04614.x>

453 NWACHUKWU, I.D.; ALUKO, RE. Structural and functional properties of food protein-  
454 derived antioxidant peptides. **Journal of Food Biochemistry**, v.43, 12761, 2019. DOI:  
455 <https://doi.org/10.1111/jfbc.12761>.

456 RAJAGOPAL, M.; WALKER, S. Envelope structures of gram-positive bacteria. **Current**  
457 **Topics in Microbiology and Immunology**, Springer, Cham. v.404, p1-44, 2017. DOI:  
458 [https://doi.org/10.1007/82\\_2015\\_5021](https://doi.org/10.1007/82_2015_5021).

459 SEBRANEK, J.G.; SEWALT, V.J.H.; ROBBINS, K.L.; HOUSER, T.A. Comparison of a  
460 natural rosemary extract and BHA/BHT for relative antioxidant effectiveness in pork  
461 sausage. **Meat Science**, v.69, p.289–296, 2005. DOI:  
462 <https://doi.org/10.1016/j.meatsci.2004.07.010>.

463 SENGKHUI, S.; KLUBTHAWEE, N.; AUNPAD, R. A novel designed membrane-active  
464 peptide for the control of foodborne *Salmonella enterica* serovar Typhimurium.  
465 **Scientific Reports**, v.13, 3507, 2023. DOI: [https://doi.org/10.1038/s41598-023-30427-](https://doi.org/10.1038/s41598-023-30427-z)  
466 [z](https://doi.org/10.1038/s41598-023-30427-z).

467 SHI, C.; LIU, M.; ZHAO, H.; LV, Z.; LIANG, L.; ZHANG, B. A Novel Insight into  
468 Screening for Antioxidant Peptides from Hazelnut Protein: Based on the Properties of  
469 Amino Acid Residues. **Antioxidants** (Basel), v.11, 127, 2022. DOI:  
470 <https://doi.org/10.3390/antiox11010127>.

471 SILVEIRA, R.F.; ROQUE-BORDA, CA.; VICENTE, E.F. Antimicrobial peptides as a feed  
472 additive alternative to animal production, food safety and public health implications :  
473 An overview. **Animal Nutrition**, v.7, p. 896-904, 2021. DOI:  
474 <https://doi.org/10.1016/j.aninu.2021.01.004>.

475 TORRES, M.D.; SOTHISELVAM, S.; LU, T.K.; DE LA FUENTE-NUNEZ, C. Peptide  
476 design principles for antimicrobial applications. **Journal of Molecular Biology**, v.431,  
477 p.3547–3567, 2019. DOI: <https://doi.org/10.1016/j.jmb.2018.12.015>.

478 TROVASLET, M.; KAPEL, R.; RAVALLEC-PLE, R.; MOUNI, F.; CLARISSE, M.;  
479 FAILLE, C.; DHULSTER, P.; GUILLOCHON, D.; VERCAIGNE, D.M. Secretagogue  
480 and bacteriosatatic active fraction derived from a peptic hydrolysate of alfalfa RuBisCO

481 small, purified subunit. **Journal of the Science of Food and Agriculture**, v.87, p.534-  
482 540, 2007. DOI: <https://doi.org/10.1002/jsfa.2754>.

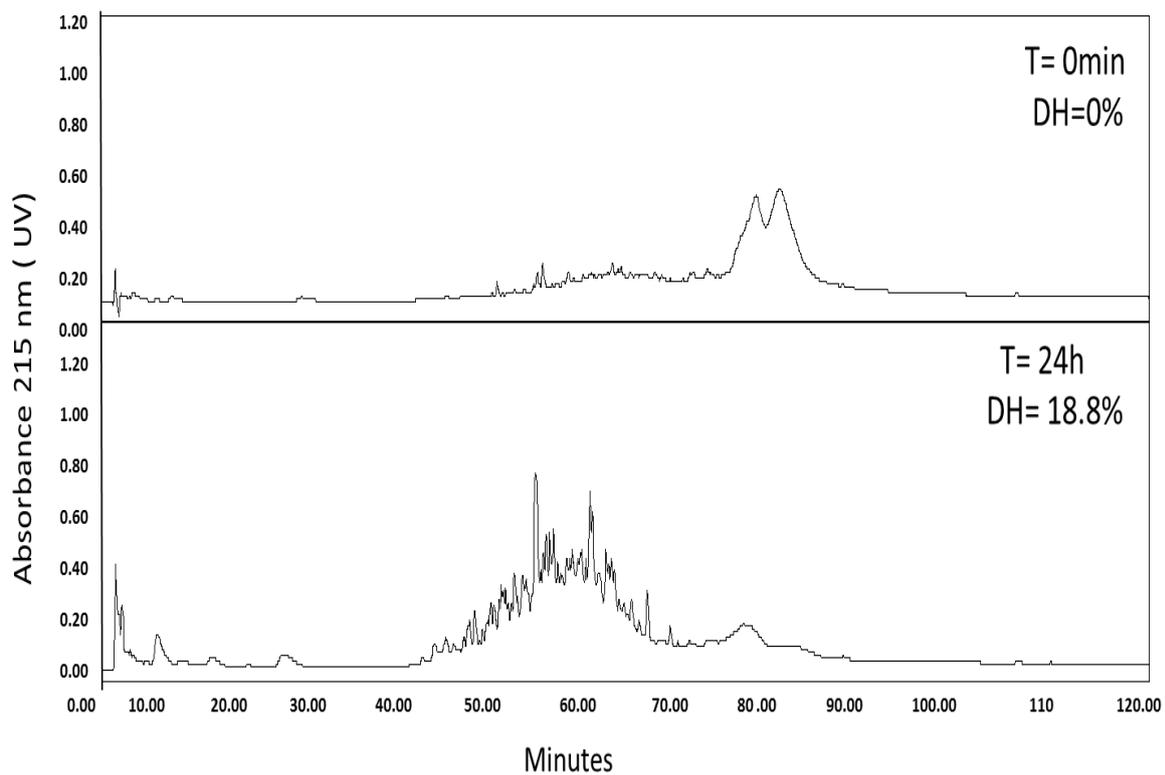
483 TU, M.; LIU, H.; CHENG, S.; MAO, F.; CHEN, H.; FAN, F.; LU, W.; DU, M. Identification  
484 and characterization of a novel casein anticoagulant peptide derived from in vivo  
485 digestion. **Food & Function**, v.10, p.2552-2559, 2019. DOI: 10.1039/c8fo02546k.

486 WANG, L.S.; HUANG, J.C.; CHEN, Y.L.; HUANG, M.; ZHOU, G.H. Identification and  
487 characterization of antioxidant peptides from enzymatic hydrolysates of duck meat.  
488 **Journal of Agricultural and Food Chemistry**, v.63, p.3437-3444, 2015. DOI:  
489 <https://doi.org/10.1021/jf506120w>.

490 WITTE, V.C.; KRAUSE, G.F.; BAILY, M.E. A new extraction method for determination 2-  
491 thiobarbituric acid values of pork and beef during storage. **Journal of Food Science**,  
492 v.35, p.582-585, 1970. DOI: <https://doi.org/10.1111/j.1365-2621.1970.tb04815.x>.

493 ZAGOREC, M.; CHAMPOMIER-VERGES, M.C. Meat microbiology and spoilage. In  
494 Lawrie' s meat science, p. 187–203, 2017. Elsevier. DOI: [https://doi.org/10.1016/B978-  
495 0-08-100694-8.00006-6](https://doi.org/10.1016/B978-0-08-100694-8.00006-6).

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508 **Figure 1.** Chromatographic profiles of RuBisCO without hydrolysis (DH=0%) and RuBisCO

509 hydrolysis at 215 nm by RP-HPLC with a C4 column at 18.8% hydrolysis degree for 24 h.

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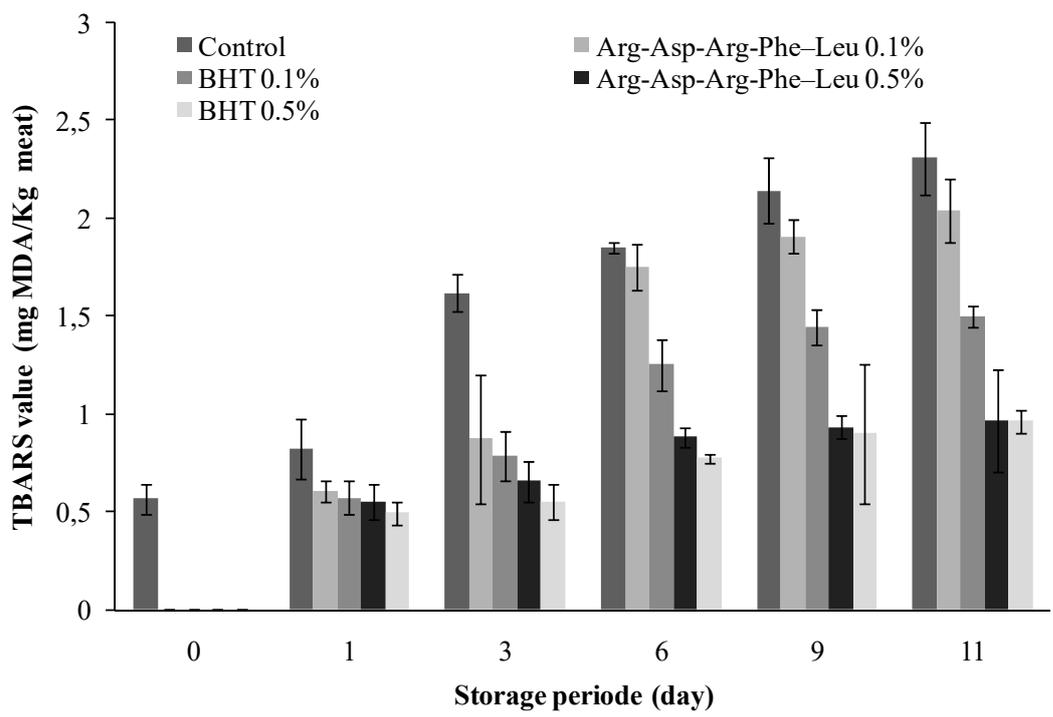
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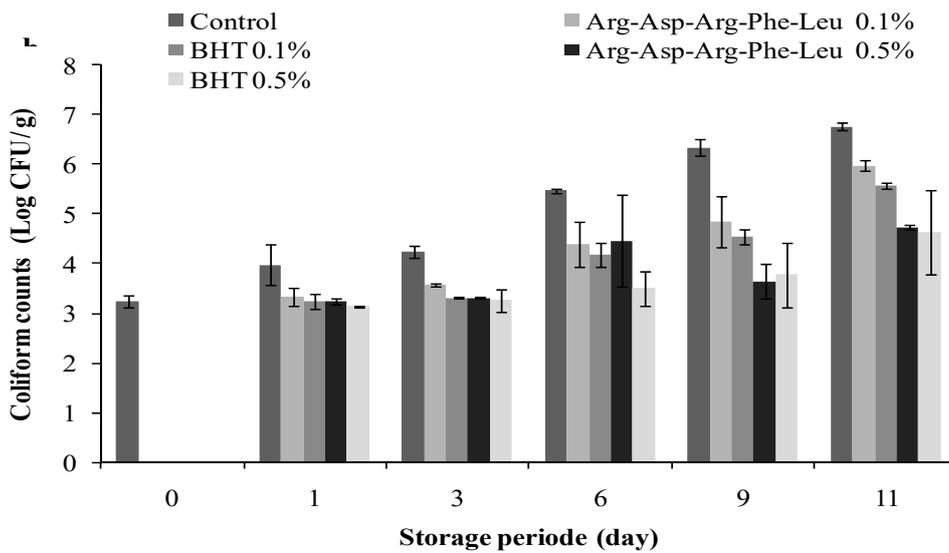
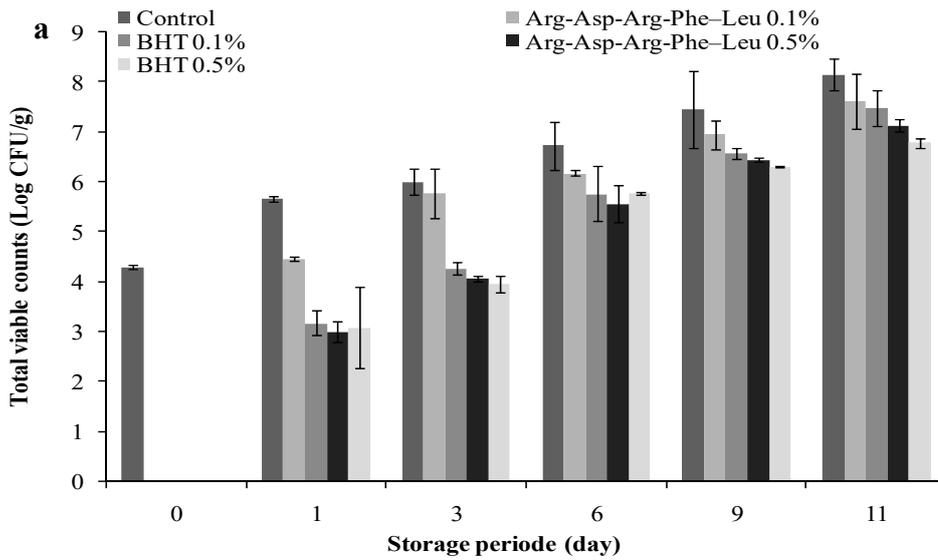


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**Figure 3.** Lipid oxidation evaluation of the meat treated with BHT and Arg-Asp-Arg-Phe-Leu peptide at concentrations of 0.1 and 0.5% (w/w) during storage under refrigeration.

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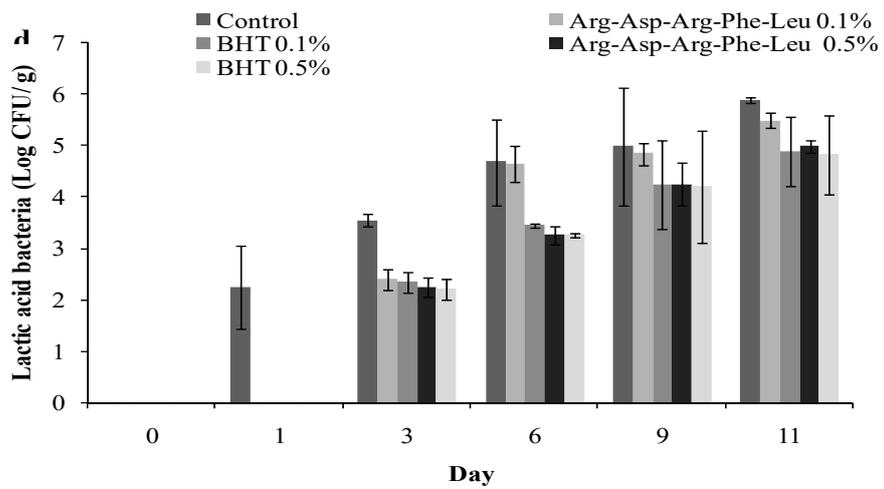
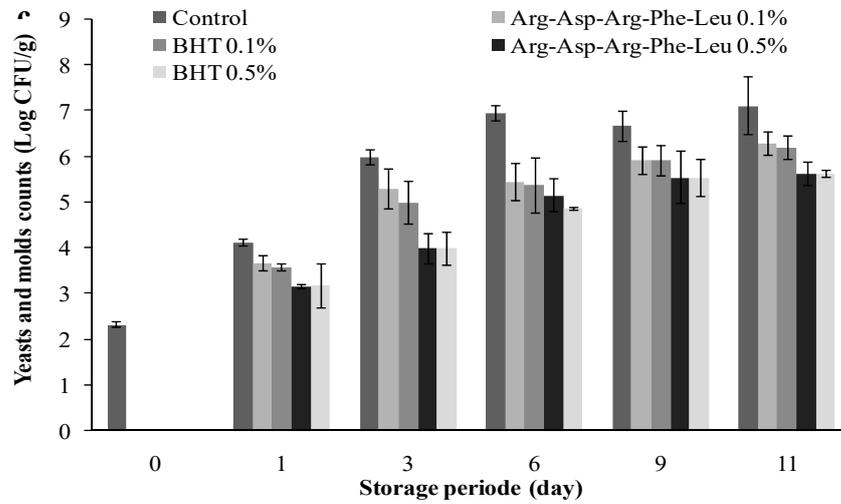
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614 **Figure 4.** Microbial changes in meat treated with BHT and Arg-Asp-Arg-Phe-Leu peptide at

615 concentrations of 0.1 and 0.5% (w/w) during storage under refrigeration. (a) Total viable

616 counts, (b) Coliform counts, (c) Yeasts and molds counts (d) Lactic acid bacteria counts.