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

Abstract – The objective of this work was to purify the small (705 Da) and hydrophilic antimicrobial Arg-Asp-Arg-Phe-Leu peptide from RuBisCO protein hydrolysate and to evaluate its effect on the microbiological and oxidative stability of beef mince during refrigeration. RuBisCO was obtained from alfafa green juice. The peptide extract was fractionated using RP-HPLC, and the active fractions were analyzed by liquid chromatography, electrospray ionization, and tandem mass spectrometry (LC-ESI-MS). Beef mince was analyzed in the following treatments: negative control, meat with two different BHT concentrations of 0.1 and 0.5% (w/w), and meat with two different Arg-Asp-Arg-Phe-Leu peptide concentrations of 0.1 and 0.5% (w/w). Lipid oxidation using the thio-barbituric acid-reactive substance (TBARS) values were significantly affected by the storage period and the concentration of bioactive peptide. Arg-Asp-Arg-Phe-Leu, a small antibacterial peptide from RuBisCO, can be isolated and purified by HPLC from alfafa green juice with retention time between 10 and 50 min, which corresponds to antimicrobial peptides. RuBisCO peptide Arg-Asp-Arg-Phe-Leu 0.5% increases oxidative stability of beef mince during refrigeration. RuBisCO peptide Arg-Asp-Arg-Phe-Leu inhibit microbial growth under refrigeration for 11 days.

Index terms: antimicrobial, antioxidant, beef meat, bioactive peptide.

Peptídeo do hidrolisado protéico de RuBisCO e sua aplicação na preservação de carne bovina

Resumo – O objetivo deste trabalho foi purificar peptídeo antimicrobiano pequeno (705 Da) e hidrofílico Arg-Asp-Arg-Phe-Leu a partir do hidrolisado proteico RuBisCO e avaliar seu efeito na estabilidade microbiológica e oxidativa de carne bovina moída durante refrigeração. RuBisCO foi obtida a partir do suco verde de alfafa. O extrato peptídico foi fracionado utilizando RP-HPLC, e as frações ativas foram analisadas por cromatografia líquida, ionização por eletrospray e espectrometria de massa em tandem (LC-ESI-MS). A carne bovina moída foi analisada nos seguintes tratamentos: controle negativo, carne com duas concentrações diferentes de BHT de 0,1 e 0,5% (p/p) e duas concentrações de peptideo Arg-Asp-Arg-Phe-Leu de 0,1 e 0,5% (p/p). Os valores de oxidação lipídica utilizando a substância reativa ao ácido tiobarbitúrico (TBARS) foram significativamente afetados pelo período de armazenamento e pela concentração do peptídeo bioativo. Arg-Asp-Arg-Phe-Leu, um pequeno peptídeo de RuBisCO, pode ser isolado e purificado por HPLC de suco verde de alfafa com tempos de retenção de 10 e 50 min, o que corresponde ao peptídeo antimicrobiano. O peptídeo Arg-Asp-Arg-Phe-Leu 0,5% aumenta a estabilidade de oxidação da carne moída durante a refrigeração. O peptídeo Arg-Asp-Arg-Phe-Leu de RuBisCO inibe o crescimento microbiano sob refrigeração durante 11 dias.

Termos para indexação: antimicrobiano, antioxidante, carne bovina, peptídeo bioativo.

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Introduction

Fresh products as beef mince contains an adequate amount of water, abundance of proteins, and essential nutrients with a pH that favors microbial growth. Therefore, meat is a highly perishable product that requires appropriate preservation (Domínguez et al., 2018). To prolong shelf life and preserve the quality of meat and its products, several synthetic additives have long been directly applied as food additives or used as processing aids (Munekata et al., 2020). Hashemi et al. (2017) cited that the use of butylated hydroxytoluene (BHT) and propyl gallate (PG) has been questioned because of the potential toxic and carcinogenic effects.

Consumer health concerns and a trend towards natural food additives, the so called clean-labeling, has driven exploring of natural antimicrobial compounds as an alternative to synthetic food additives (Lorenzo et al., 2018). The advancement in bioactive peptide research has showed that the peptides derived from food proteins could be natural candidates to replace the synthetic compounds (Chakrabarti et al., 2018). Indeed, antimicrobial peptides of industrial waste could be used as preservatives (Lafarga & Hayes, 2014). To date, several animal peptides such as lysozyme, lactoferrin, and nisin are approved for application in meat and its products (Silveira et al., 2021).

Recently, the food industries have been interested in peptides derived from agro-industrial waste, such as the green juice derived from the bio-refinery of alfalfa (*Medicago sativa*), often considered a source of pollution, which is an excellent protein-rich source and has become an alternative for peptides generation with great interest for food formulation (Hadidi et al., 2019).

Ribulose-1,5-bisphosphate carboxylase oxygenase, most commonly known as RuBisCO, is the most abundant plant protein and might be a promising candidate for use in food applications, due to its unique characteristics, favorable nutritional profile, beneficial bioactivities, and desirable physical attributes (Grácio et al., 2023). Several studies showed that RuBisCO contains several bioactive peptides compared to commonly consumed food proteins, except milk proteins (Corrêa et al., 2023).

In recent studies, antibacterial and antioxidant peptides derived from the enzymatic hydrolysis of alfalfa RuBisCO were isolated and identified (Kobbi et al., 2015, 2017). The antibacterial peptide Arg-Asp-Arg-Phe-Leu was previously identified from protein hydrolysates of alfalfa RuBisCO prepared with pepsin (Kobbi et al., 2015). Arg-Asp-Arg-Phe-Leu, a small (705 Da) peptide with one positive charge at pH 7, belongs to the great subunit of the RuBisCO, which has a large antibacterial spectrum against Gram-positive and Gram-negative bacteria (Kobbi et al., 2015). This bioactive peptide has several attractive properties and attributes that make it promising to be used as preservative in the storage and distribution of various meat products.

The objective of this work was to purify the small (705 Da) and hydrophilic antimicrobial Arg-Asp-Arg-Phe-Leu peptide from RuBisCO protein hydrolysate and to evaluate its effect on the microbiological and oxidative stability of beef mince during refrigeration.

Materials and Methods

The purification of RuBisCO was carried out by a simple method based on pH and solvent effect, according to Kobbi et al. (2017). Green juice samples were produced from the dehydration process of alfalfa leaves. Alfalfa was harvested, milled, and cold pressed, resulting in a paste that was dried and granulated in a fluid bed at low temperature to obtain a heterogeneous dried green juice, similar to what occurs in large-scale production.

In order to isolate a soluble fraction, 100 g of alfafa green juice was solubilized in 1 L of distilled water or 1 L of ammonia (NH_4OH) at 0.1 M and pH 11. Suspensions were centrifuged at 10,000 g for 20 min at 4°C to eliminate the green protein and all debris. The RuBisCO containing base 1 at pH 10 was repurified to retrieve the second supernatant 2. Base 2 was repurified again to recover supernatant 3. After three successive centrifugations at pH 10, all insoluble fractions were removed, and, hence, three supernatants were summed up to recover all RuBisCO.

Proteins were then precipitated by the acidification of the medium at pH 3 using 10 mM of formic acid, followed by a last centrifugation step at 10,000 g at 4°C for 20 min. The precipitate was washed by ultrapure water and RuBisCO was then lyophilized. A 10% (w/v) aqueous solution of the lyophilized RuBisCO was adjusted to pH 10 and then centrifuged at 10,000 g for 5 min. The supernatant was adjusted to optimal pH 3 and temperature of 37°C for pepsin (Merck KGaA, Darmstadt, Germany).

The hydrolysis reaction was started by the addition of the enzyme at a 1:100 (U/mg) enzyme-protein ratio. The enzymatic reaction was conducted at 37°C and pH 3 for 24 h. The hydrolysis reactions were stopped after 24 hours by increasing the pH to 10 with 1 M NaOH and then stored at -20°C for further use. The degree of hydrolysis (DH) was defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds of protein substrate. DH was determinated by the OPA (o-phthaldialdehyde) method using leucine as standard (Nielsen et al., 2001).

The RuBisCO hydrolysate was analyzed using Reversed Phase HPLC (RP-HPLC) on a C4 column Vydac (Grace Corporate, Columbia, MD, USA) according to Trovaslet et al. (2007) with slight modifications. The liquid chromatographic system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector (Waters Corporation, Milford, MA, USA). Spectral and chromatographic data were stored on a NEC Image 466 Image 466 computer. Millennium software was used to plot, acquire, and analyze chromatographic data.

The mobile phase composed of water/trifluoroacetic acid (1000:1, v/v) was used as eluent A, while acetonitrile/trifluoroacetic acid (1000:1, v/v) was used as eluent B. The flow rate was 0,6 ml/min. Samples were filtered through 0.22 mm filters and then injected. The gradient applied was 0 to 10 min, 100% A; 25 to 95 min, 21 to 81% B; 96 to 110 min, 100 to 0% B; and the HPLC system were equilibrated for 10 min with 100% A. On-line UV absorbance scans were performed between 215 and 325 nm at a rate of one spectrum per second with a resolution of 1.2 nm. Chromatographic analyzes were completed with Millennium software.

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

The molecular mass and peptide sequencing were done in positive ion mode using Electrospray ionisation-mass spectrometry (ESI-MS) and the tandem mass spectrometry (MS/MS), respectively. ESI mass spectrometry was performed using the triple quadrupole instrument Applied Biosystems API 3000 (Sciex, Framingham, MA, USA) equipped with an electrospray ion source. The system is controlled by the Analyst Software 1.4, allowing the control of the spectrometer, the analysis and the processing data. Interpretations of spectra MS-MS were made with the Bioanalyst software.

The freeze-dried samples from RP-HPLC were dissolved in acetonitrile/water (20/80; v/v) containing 0.1% formic acid for the positive mode. The solution was injected (nebulised) uninterrupted, by a Model 22 pump (Harward Apparatus, Holliston, MA,USA) with a flow rate of 5 µl/min. The potential of ionization was of 5,000 V in positive mode. At the time of the recording of the spectrum, 30 scans on average were added for each spectrum. The gases used were up to 99% pure nitrogen and air and produced by a Jun-Air 4000-40M compressor and a Whatman model 75-72 nitrogen generator (Global Life Sciences Solutions, Marlborough, MA, USA). The polypropylene glycol (PPG) was used for the calibration and the optimization of the machine. The peptide sequence was determined from the CID spectrum of the protonated analyse $[M+H]^+$ by MS/MS experiments. Peptide sequences were done using the Bioanalyst software.

The antimicrobial peptide Arg-Asp-Arg-Phe-Leu was chemically synthesized by Fmoc solid phase synthesis, according to the method described by Fields & Noble (1990). Arg-Asp-Arg-Phe-Leu peptide was synthesized on a Fmoc-Arg (Pbf)-WangTentagel S resin, 0.25 mmol/g, 50 µmol scale (Iris Biotech GmbH, Marktredwitz, Germany) using a peptide synthesizer (Intavis Bioanalytical Instruments AG, Köln, Germany) and standard Fmoc/tert-butyl.

Activation of the amino acids (10 eq) was performed separately prior to the coupling step using a mixture of TBTU/HOBt/DIEA: 10 eq/10 eq/30 eq in DMF. A capping step was performed after each coupling with a mixture of Ac2O/NMM/DMF: 5/10/75 v/v/v. At the end of the synthesis, the Fmoc protecting group was

removed using 20% piperidine in DMF. The resin was washed with DMF (4 × 2 min) and CH2Cl2 (4 × 2 min) and dried in vacuum. Deprotection and cleavage of the peptide from the resin was performed with a mixture of TFA/TIS/H2O at room temperature. The mixture was precipitated in cold diethyl ether. The peptide was collected by centrifugation, dissolved in deionized water and lyophilized. Arg-Asp-Arg-Phe-Leu was purified by RP-HPLC.

The beef mince was composed of commercial beef, containing 0.28% (w/w) of salt, 14% (w/w) of fat, and about 18% of proteins. Salt, 70% NaCl and 30% KCl, was added at a rate of 1.5% to the beef mince to encourage slow growth of spoilage microorganisms. The sample was subdivided into the following treatments: negative control, meat without additive; meat with two different BHT concentrations, of 0.1 and 0.5% (w/w); and the meat with two different Arg-Asp-Arg-Phe-Leu peptide concentrations, of 0.1 and 0.5% (w/w). The meat sample preparation was realized under strong aseptic conditions to avoid contamination. Samples of 10 g of beef mince were placed in plastic foam meat trays, wrapped with film polyethylene and kept at 4°C for 11 days. Four samples were taken from each treatment at fixed day in order to evaluate their oxidation and the potential microbial contamination.

Lipid oxidation was evaluated using the thiobarbituric acid-reactive substance (TBARS) as previously described by Witte et al. (1970).

Microbial count was carried out at days 0, 1, 3, 6, 9, and 11 of storage at 4°C according to the following procedure: 45 mL sterilized peptone solution (0.1% w/v) was added to each five grams of beef patty in a sterile stomacher bag, then homogenized. Decimal dilutions up to 105 prepared from the initial concentration of 100 mg/mL and 100 µL of the appropriate dilutions were spread out on different selective media. Total viable counts were determined using plate count agar (PCA), incubated for 48 hours at 30°C (ISO, 2013). Potato dextrose agar (PDA) for yeasts and molds counts, was incubated for 24 hours at 37°C (ISO, 2008). Man, Rogosa and Sharpe (MRS) agar for lactic acid bacteria, incubated for 72 hours at 30°C, (ISO, 1998) and violet, red bile lactose (VRBL) agar for coliform counts (ISO, 2006) were used after incubation at 37°C for 24 hours. Microbiological count was expressed as the log10 of colony-forming units per gram of patty (log10 CFU/g).

All data were submitted to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Test. The data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 10.0 (Chicago, Illinois, USA). Differences were considered significant at $p<0.05$.

Results and Discussion

Generated hydrolysate (DH = 18%) was mainly intermediate peptides that were produced from the beginning of hydrolysis and disappearing gradually as hydrolysis advanced (Figure 1). The chromatographic profile (Figure 2 A) showed the peptide population with retention time between 10 and 50 min, which corresponded to antimicrobial peptides. According to the mass spectrometry analyses (Figure 2 B), Arg-Asp-Arg-Phe-Leu antimicrobial pentapeptide was identified. The minimum inhibitory concentration (MIC) of the purified peptide varied between 2.17±0.1 mM for *Listeria innocua* and *Bacillus subtilis*, and 2.83±0.2 mM for *Escherichia coli* and *Micrococcus luteus*. Peptides showed an outstanding antibacterial activity against both Gram-positive and Gram-negative bacteria by damaging the cell membrane (Tu et al., 2019; Bi et al., 2020).

Significant changes in beef mince occurred over the sampling period when Arg-Asp-Arg-Phe-Leu peptide

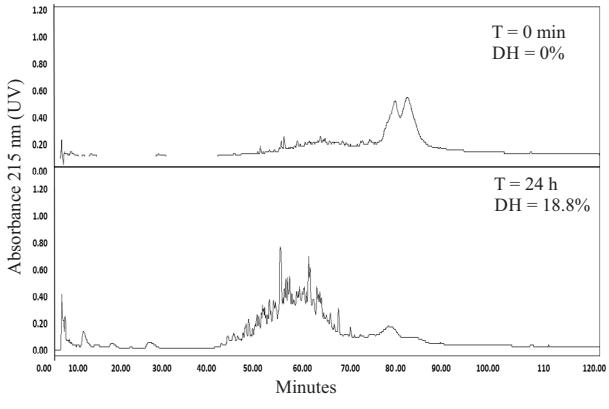

Figure 1. Chromatographic profiles of RuBisCO without hydrolysis (DH = 0%) and RuBisCO hydrolysis at 215 nm by RP-HPLC with a C4 column at 18.8% hydrolysis degree for 24 hours.

was added (Figure 3). The TBARS values increased throughout the 11-day assay for all treatments evaluated. This fact was clearly marked for the control sample with the highest TBARS values than all tested conditions by the end of storage. While the TBARS values of the meat supplemented with either 0.5 or 0.1% (w/w) Arg-Asp-Arg-Phe-Leu peptide were significantly lower than the control treatment. Moreover, the efficiency in ground meat preservation increased proportionally with peptide concentration. But this difference was clear from day 6 because no significant effect between the treated groups has been observed during the first three days of the experiment, but their TBARS values were significantly lower than the control. From day 6 on, the power antioxidant of Arg-Asp-Arg-Phe-Leu at 0.5% (w/w) was higher than the other two treated groups, peptide at 0.1% and BHT at 0.1%. Thus, the peptide at 0.5% (w/v) can protect beef meat against lipid oxidation and extend its storage time.

The TBARS values of the meat supplemented with either peptide 0.5% or BHT 0.5% (w/v), used as reference, have no significant difference and were similar after nine days of storage at 4°C. Consequently, the BHT 0.5% (w/v) and Arg-Asp-Arg-Phe-Leu 0.5% (w/w) reduced the lipid oxidation on meat in 60% compared to the control. This tendency continued until 11 days. At the end of the experiment, the BHT 0.5 (w/w) and Arg-Asp-Arg-Phe-Leu 0.5% (w/w) had no significant difference. These results confirm the results obtained by Kobbi et al. (2017), which revealed that the alfalfa RuBisCO hydrolysate (DH = 18.8%) exhibited strong antioxidant abilities, including the prevention of linoleic acid oxidation, reduction of ferric ion, and stability determined by ABTS method.

Therefore, green juice derived from bio-refinery of alfalfa offers a promising source of antioxidant peptides of interest for various functional food applications. The antioxidant activity of this peptide was probably due to its low molecular weight (705 Da) and the presence of aromatic residue phenylalanine (Phe) which can quench free radicals by direct electron transfer to act as an antioxidant (Geng et al., 2023). Peptides with highly potent inhibitory activity have hydrophobic amino acids as leucine (Leu), at the N-terminal position in the sequences (Shi et al., 2022).

The microbial count increased proportionally with the storage time and reached the highest values at the end of the chill period. Among the experimental groups, the negative control group showed the most rapid increase in the number of total microorganisms, followed by samples treated with Arg-Asp-Arg-Phe-Leu peptide and BHT (Figure 4 A). The coliform count on a VRBL medium shows that no significant difference has been observed among the groups treated with additives during the first six days (Figure 4 B). But after day 6, the group with Arg-

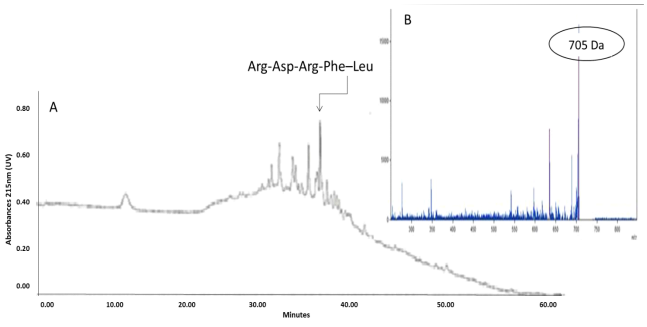

Figure 2. Reverse-phase HPLC profile of Arg-Asp-Arg-Phe-Leu peptide prepared using precipitation from RuBisCO hydrolysate with 2 M of NaCl and at pH 7.5 (A). ESI-MS spectrum of the Arg-Asp-Arg-Phe-Leu peptide, MS measurements were performed in positive ion mode using electrospray ionization (B).

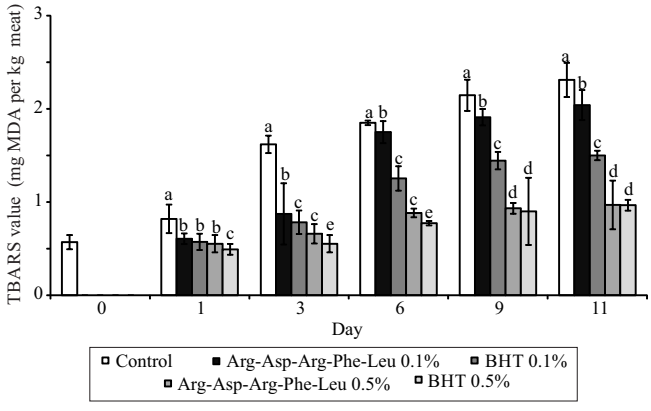

Figure 3. Lipid oxidation evaluation of the meat treated with BHT and Arg-Asp-Arg-Phe-Leu peptide at concentrations of 0.1 and 0.5% (w/w), respectively, during storage under refrigeration. Same letter above the bars showed the statistical differences ($p>0.05$) between the tested samples and the standard.

Asp-Arg-Phe-Leu peptide 0.5% (w/w) showed more efficiency when compared with that of BHT 0.5% (w/w). Therefore, this peptide can reduce 32% the number of total coliforms in meat compared with the control treatment.

For the yeasts and molds, the most effective additives were Arg-Asp-Arg-Phe-Leu 0.5% (w/w) and BHT 0.5% (w/w) (Figure 4C). These effects were similar to BHT 0.5% (w/w), because they had no significant difference. On the other hand, the groups treated with BHT 0.1% (w/w) and Arg-Asp-Arg-Phe-Leu 0.1% (w/w) are less effective and they presented no significant difference with the yeasts and molds counts at day 11, compared with the control group.

During the first three days, no lactic acid bacterial proliferation has been observed for the groups treated with additives, while the number of lactic acid bacteria was increased in the control group (Figure 4 D). After six days, the groups treated with peptide 0.5%, BHT 0.1%, and BHT 0.5% showed a significant difference compared with the control group. Once again, the most effective groups were the Arg-Asp-Arg-Phe-Leu 0.5% (w/w) and BHT 0.5% (w/w) ones.

Zagorec & Champomier-Vergès (2017) reported the impact of antimicrobial peptides on meat and its products. Antimicrobial peptides have unique structural characteristics, such as their exclusive tendency to adapt to various structural changes when they come into contact with membranes (Torres et al., 2019). The cationic peptides exert antibacterial activity by interacting with a negatively charged bacterial membrane to increase permeability, which leads to lysis, releasing cell content. Upon approaching the cytoplasmic membrane through electrostatic interaction with the microbial membrane, antimicrobial peptides bind to the microbial membrane and interact with the anionic components of the plasma membrane (Rajagopal & Walker, 2017).

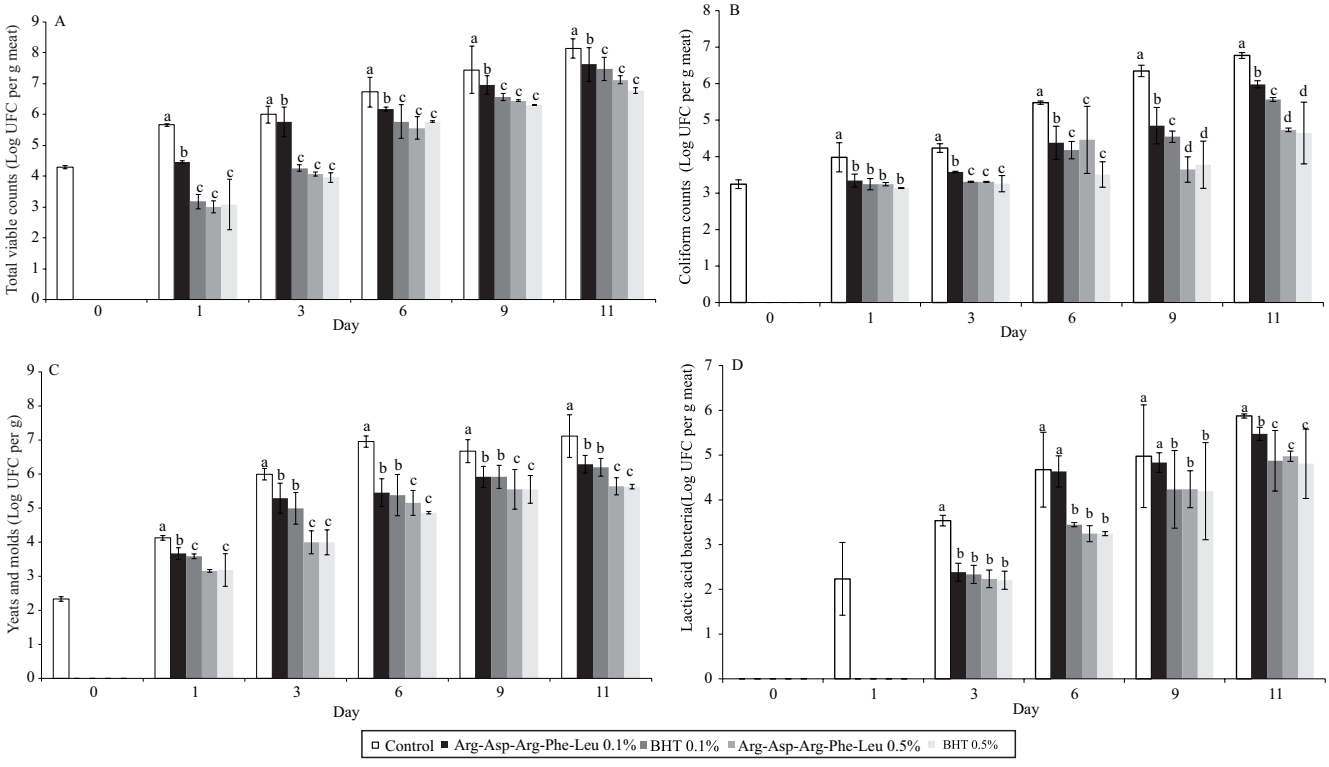

Figure 4. Microbial changes in meat treated with BHT and Arg-Asp-Arg-Phe-Leu peptide at concentrations of 0.1 and 0.5% (w/w), respectively, during storage under refrigeration. Total viable counts (A), Coliform counts (B), yeasts and molds counts (C), and lactic acid bacteria counts (D). Same letter above the bars showed the statistical differences ($p>0.05$) between the tested samples and the standard.

Conclusions

1. Arg-Asp-Arg-Phe-Leu, a small antibacterial peptide from RuBisCO, can be isolated and purified by HPLC from alfafa green juice with retention time between 10 and 50 min, which corresponds to antimicrobial peptides.

2. RuBisCO peptide Arg-Asp-Arg-Phe-Leu 0.5% increases oxidative stability of beef mince during refrigeration.

3. RuBisCO peptide Arg-Asp-Arg-Phe-Leu inhibit microbial growth under refrigeration for 11 days.

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