Research, Society and Development, v. 9, n. 12, e27591210772, 2020
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v9i12.10772

Antioxidant activity, inhibition of angiotensin I converting enzyme (ACE) and antibacterial activity of buffalo caseinate protein hydrolysates and their fractions

Atividade antioxidante, inibição da enzima conversora de angiotensina I (ECA) e atividade antibacteriana de hidrolisados proteicos de caseinato de búfalo e suas frações

Actividad antioxidante, inhibición de la enzima convertidora de angiotensina I (ECA) y actividad antibacteriana de hidrolizados de proteína de caseinato de búfalo y sus fracciones

Received: 12/01/2020 | Reviewed: 12/08/2020 | Accept: 12/18/2020 | Published: 12/24/2020

Wellington Leal dos Santos
ORCID: https://orcid.org/0000-0001-6257-7743
Universidade Federal de Pernambuco, Brazil
E-mail: wellingtonleal16@gmail.com
Thailan Arlindo da Silva
ORCID: https://orcid.org/0000-0003-2761-4013
Universidade Federal do Agreste de Pernambuco, Brazil
E-mail: thailanarlindo590@gmail.com
Patricia Lins Azevedo do Nascimento
ORCID: https://orcid.org/0000-0002-6335-0193
Universidade de Pernambuco, Brazil
E-mail: patricialanascimento@hotmail.com
Rosângela Estevão Alves Falcão
ORCID: https://orcid.org/0000-0002-7693-4630
Universidade de Pernambuco, Brazil
E-mail: rosangela.estevao.falcao@hotmail.com
João Tiago Correia Oliveira
ORCID: https://orcid.org/0000-0001-7469-5106
Universidade Federal do Sul e Sudeste do Pará, Brazil
E-mail: joaotiago@zootecnista.com.br
Keila Aparecida Moreira
ORCID: https://orcid.org/0000-0002-7715-9285
Universidade Federal do Agreste de Pernambuco, Brazil

E-mail: moreirakeila@hotmail.com

Abstract

In the present study, buffalo milk caseinate hydrolysates produced by bromelain, neutrase, papain and trypsin were ultra-filtered and different fractions were assessed for antioxidant, inhibition of angiotensin converting enzyme and antimicrobial activity. Biological potential was assessed by a number of metrics: ability to remove radicals of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyls; copper and iron chelation; antidiabetic properties; antihypertensive assay; and antibacterial activity against *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19114, *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923 strains. The tests for scavenging of hydroxyl radicals and DPPH revealed a greater potential in the 3–10 kDa fractions. Iron chelation activity >70% was observed in all the fractions, including <3 kDa. Copper chelation was >60% in fractions >10 kDa. α-Amylase inhibition and antihypertensive activity was optimal in the <3 kDa fraction. Antibacterial activity ranged between 3.28 and 100% inhibition against microorganisms tested, the fraction <3 kDa showed a greater inhibitory potential. The antihypertensive activity of fractions ranged between 39.35 and 89.58%. All treatments were able to produce hydrolysates and fractions with biological potential and, so the ultrafiltration method proved to be effective in the separation of peptides with different molar masses and potential use in the food or pharmaceutical industry.

Keywords: Ultrafiltration; Buffalo milk; Functional food.

Resumo

No presente estudo, os hidrolisados do caseinato de leite de búfala produzidos por bromelina, neutrase, papaína e tripsina foram ultrafiltrados, e diferentes frações foram avaliadas quanto à atividade antioxidante, inibição da enzima conversora de angiotensina I e antimicrobiana. O potencial biológico foi avaliado através de uma série de atividades: capacidade de remover radicais de 2,2'-azino-bis (ácido 3-etilbenztiazolina-6-sulfônico), 2,2'-difenil-1-picrylhydrazyl (DPPH) e hidroxilas; quelação de cobre e ferro; propriedades antidiabéticas; inibição da enzima conversora de angiotensina; e atividade antibacteriana contra *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19114, *Salmonella typhimurium* ATCC 14028 e cepas de *Staphylococcus aureus* ATCC 25923. Os testes para sequestro dos radicais hidroxila e DPPH revelaram um maior potencial nas frações de 3-10 kDa. A atividade de quelação do ferro >70% foi observada em todas as frações, incluindo <3 kDa. A quelação do cobre foi

>60% nas frações >10 kDa. A inibição da α-amilase e atividade anti-hipertensiva foi ótima na fração <3 kDa. A atividade antibacteriana variou entre 3,28 e 100% de inibição contra os microrganismos testados. A fração <3 kDa mostrou um potencial inibitório maior. A atividade anti-hipertensiva das frações variou entre 39,35 e 89,58%. Todos os tratamentos foram capazes de produzir hidrolisados e frações com potencial biológico e, portanto, o método de ultrafiltração prova ser eficaz na separação de peptídeos com diferentes massas molares e uso potencial na indústria alimentícia ou farmacêutica.

Palavras-chave: Ultrafiltração; Leite de búfalo; Alimentos funcionais.

Resumen

En el presente estudio, los caseinatos de leche de búfala producidos por bromelina, neutrasa, papaína y tripsina fueron ultrafiltrados y se evaluó la actividad antioxidante y antimicrobiana de diferentes fracciones. El potencial biológico se evaluó mediante una serie de mediciones: capacidad de eliminar los radicales del 2,2'-azino-bis (ácido 3-etilbenctiazolín-6-sulfónico), 2,2'-difenil-1-picrilhidrazilo (DPPH) e hidroxilos; quelación del cobre y el hierro; propiedades antidiabéticas; inhibición de la enzima convertidora de angiotensina; y actividad antibacteriana contra las cepas de *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19114, *Salmonella typhimurium* ATCC 14028 y *Staphylococcus aureus* ATCC 25923. Las pruebas de eliminación de radicales hidroxilo y DPPH revelaron un mayor potencial en las fracciones de 3-10 kDa. Se observó una actividad de quelación del hierro >70% en todas las fracciones, incluyendo <3 kDa. La quelación del cobre fue >60% en las fracciones >10 kDa. α La inhibición de la amilasa y la actividad antihipertensiva fue óptima en la fracción <3 kDa. La actividad antibacteriana osciló entre 3,28 y 100% de inhibición contra los microorganismos probados, la fracción <3 kDa mostró un mayor potencial inhibitorio. La actividad antihipertensiva de las fracciones osciló entre el 39,35 y el 89,58%. Todos los tratamientos fueron capaces de producir hidrolizados y fracciones con potencial biológico, por lo que el método de ultrafiltración demostró ser eficaz en la separación de péptidos con diferentes masas molares y su posible uso en la industria alimentaria o farmacéutica.

Palabras clave: Ultrafiltración; Leche de búfalo; Alimentos funcionales.

1. Introduction

The buffalo (*Bubalus bubalis*) is considered to be a domesticated animal on several continents (FAO, 2017), namely Asia (India, Pakistan and China), Europe (Italy), Africa

(Egypt) and South America (mainly in Argentina and in Brazil). According to the Bulletin International Dairy Federation (2017) the world production of bubaline milk has recently been growing by approximately 3.3%, higher than that of bovine milk production.

Buffalo milk contains between 3.8% and 4.5% more protein than bovine milk, although the buffalo micelle is larger in relation to bovine, both have about 92% similarity (Trommelen, et al., 2018; Fangmeoer, et al., 2019; Pietrzak-Fiecko & Kamelska-Sadowska, 2020).

Intact casein is a source of essential amino acids for the growth and maintenance neonates; however, its structure also contains a series of encrypted peptides that, when released by enzymatic hydrolysis or fermentation, possess a range of biological functionalities (Kumar, et al., 2016; Trommelen, et al., 2020). Peptides can be used as additives in the formulation of functional foods, nutraceuticals and pharmaceuticals (Fosgerau & Hoffmann, 2015; Heal, et al., 2019; Sawicka, et al., 2020) and do not present only some negative side effects (Nielsen, et al., 2017).

Various biological activities of bioactive peptides derived from enzymatic hydrolysis of bubaline casein have been described, especially in the beta fraction, including inhibition of angiotensin-converting enzyme (Abdel-Hamid, et al., 2017; Sujarwanta, et al., 2018) and antioxidant (Shazly, et al., 2017; Shazly, et al., 2019) and antimicrobial activities (Nielsen, et al., 2017; Zhao, et al., 2020).

The research on buffalo milk protein is limited mainly to whey proteins and, to a lesser extent, studies of the hydrolysis of buffalo milk casein (Abdel-Hamid, et al., 2017; Shazly, et al., 2017), representing about 1% of all studies, few studies are aimed at characterizing the biological activities of bioactive peptides derived from buffalo milk protein submitted to the action of several enzymes.

Although the biological potential of peptides derived from milk casein has been reported, commercial production of these peptides has been limited by the lack of suitable technologies, however, the ultrafiltration technique employing shear membrane can be used to separate peptides from different pasta bands (Kumar, et al., 2016). Therefore, the objective of this study was to hydrolyse buffalo milk casein using different proteolytic enzymes, fractionate by ultrafiltration and investigate its antioxidant, antidiabetic and antibacterial properties.

2. Materials and Methods

2.1 Materials

Bromelain, trypsin, papain, 2,2'-diphenyl-1-picrylhydrazyl,2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, α-Glucosidase, α-amylase, N-hydrate Hipuril-His-Leu, angiotensin-converting enzyme rabbit were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and neutral protease were from Novozymes (Denmark).

2.2 Production and ultrafiltration of bubaline casein hydrolysates

Buffalo milk was obtained from a herd in the city of Ribeirão, Pernambuco, Brazil. Bubaline sodium caseinate was produced as described by Egito et al. (2007). For hydrolysate production, caseinate protein was solubilized (1% w/v) in sodium phosphate buffer 0,1M and treated with: bromelain (E.C. 3.4.22.33, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) at 60 °C and pH 6.5; papain at 50 °C and pH 6.5; trypsin at 37 °C and pH 8.0; or neutral protease (Novozymes, Denmark) at 50 °C and pH 7.5. In each case, the enzyme:substrate ratio was 1:100 (v/v) and the reaction was for 4 hours. The hydrolytic reaction was terminated by heating to 100 °C for 10 minutes and hydrolysates were stored at −20 °C until needed. Analytical determinations were performed in Laboratory of Microbiology, Enzyme Technology and Bioproducts located in Laboratorial Center of Support to the Research of Federal University of Agreste of Pernambuco.

The hydrolysates were sequentially fractionated by centrifugal ultrafiltration using Amico ® Ultra-15 units (Millipore Ireland Ltd, Tulla Green, Ireland) with different molecular weight cut-offs for 60 minutes at 4 °C and 4000 *g*, as described previously by Kumar et al. (2016). Briefly, the whole hydrolysates (H0) were passed through a 10 kDa membrane to produce an H1 fraction (> 10 kDa) and a filtrate (≤ 10 kDa). The filtrate was then passed through a 3 kDa cut-off membrane to produce a retained H2 fraction (3 ≥ n ≤ 10 kDa) and an H3 filtrate fraction (≤ 3 kDa). All fractions had their volumes completed to 15 mL using deionized water.

2.3 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging (HRS) activity was determined as described by Bamdad, et al. (2017) and absorbance was measured at 340 nm using a microplate reader (UVM 340 Microplate Reader, ASYS Hitech GmbH, Salzburg, Austria). Hydroxyl radical scavenging activity was calculated using the equation:

$$HRS\ (\%)=\left(\frac{A_{sample}-A_{degraded}}{A_{not\ degraded}-A_{degraded}}\right)*100$$

Where, the degraded groups contain all the reagents involved in the reaction, by the use of water replacing the sample. The non-degraded group corresponds to the sample solutions of the group degraded by the use of water replacing H_2O_2.

2.4 DPPH (2,2'-diphenyl-1-picrylhydrazyl) radical scavenging assay

DPPH radical scavenging activity was determined as described by Duan, et al. (2006) and absorbance was measured at 517 nm. DPPH radical scavenging ability was calculated using the equation:

$$DPPH\ (\%)=\left[1-\left(\frac{A_{sample}-A_{white\ sample}}{A_{Control}}\right)\right]*100$$

Where, A_{sample} is the absorbance of the sample, $A_{sample\ blank}$ is the absorbance of the sample without the DPPH, and $A_{Control}$ is the absorbance of the control (DPPH solution without sample).

2.5 ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

Scavenging of the cationic radical $ABTS^{+\bullet}$ as assessed by Hernández-Ledesma et al., (2007). Fifty µL of sample was mixed with 950 µL a diluted solution in phosphate buffer 5 mM, pH 7.0 of $ABTS^{+\bullet}$ and the reaction mixture was incubated for 10 minutes in the dark and

at room temperature (23 °C). Absorbance was measured to 734 nm and ABTS radical elimination activity was calculated according to the equation:

$$\text{ABTS (\%)} = \left[\left(\frac{A_{control}-A_{sample}}{A_{control}}\right)\right] * 100$$

Where, A_{sample} is the absorbance of samples, and $A_{Control}$ is the absorbance of the negative control containing phosphate buffer.

2.6 Chelation of Fe^{2+} and Cu^{2+} by hydrolysed peptide fractions

Iron chelating activity (ICA) was measured as described by Sánchez-Vioque et al. (2012) while copper chelation was measured as described by Saiga (2003). The percentage of chelation was determined according to the equation:

$$\text{ICA (\%)} = \left[\frac{(A_{Control}-A_{Sample})}{A_{Control}}\right] * 100$$

2.7 Antidiabetic activity

α-Glucosidase (GI%) and of α-amylase (AI%) inhibition was determined as described by Kim, Wang, & Rhee (2004). The absorbance was measured at 410 nm and 540 nm, respectively. Inhibition was calculated according to the equation:

$$\text{Antidiabetic activity (\%)} = \left[\frac{(A-B)}{A}\right] * 100$$

Where "A" is the optical density of the control (lacking hydrolysate) and "B" is the optical density of the reaction in the presence of hydrolysate.

2.8 *In vitro* antihypertensive assay

Inhibition of angiotensin-converting enzyme (ACE) was evaluated using an adaptation of the methods of Zhang et al.(2009), using N-hydrate Hipuril-His-Leu (HHL) as a substrate. Percent inhibition was calculated according to equation:

$$\text{ACE inibition } (\%) = \left[\frac{A-B}{A-C}\right] * 100$$

Where: "A" is the absorbance of the reaction containing ACE and HHL; "B" is the absorbance of the reaction containing hydrolysate, ACE and HHL; and "C" is the absorbance of the HHL tested.

2.9 Antibacterial activity

The antibacterial potential of fractions and total hydrolysates was determined by the turbidity method described by Wu et al. (2013). The tests were carried out in sterile 96-well plates. *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19114, *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923 strains were grown in tryptone soy broth (TSB) for 12 hours at 37 °C. Reaction mixtures contained 100 µL of peptide fraction, 90 µL of Mueller-Hinton broth and 10 µL of bacterial suspension (10^6 CFU/mL). The positive control containing Mueller-Hinton broth with the addition of chloramphenicol (100 mg/L) and the negative control that contained 100 µL of sterile water was tested under the same conditions. Readings were made in a microplate reader at 600 nm, and each test was performed in triplicate. Inhibitory fractions were considered to be those that produced >1% inhibition.

2.10 Statistical analysis

All biological activity analyses were repeated three times and the data expressed as means with standard errors. Differences were evaluated by one-way analysis of variance (ANOVA) followed by evaluation of the differences between means with the Tukey multiple range test with a 95% confidence level using SISVAR version 5.6.

3 Results and Discussion

Among the total hydrolysate and the peptide fractions obtained from enzymatic digestion with bromelain (HFB), the major hydroxyl radical scavenging activity was found in the H2 fraction (44.07 ± 1.97%), followed by H0, H1 and H3 (Table 1). Fractions H0 and H1 exhibited equal activities while the differences between the other fractions were statistically significant ($p < 0.05$).

In the papain (HFP) and trypsin (HFT) samples, H3 (5.54 ± 4.63%) and H2 (5.59 ± 2.52%), respectively, exhibited the highest hydroxyl radical scavenging activity. In the neutral protease (HFN) samples, the highest hydroxyl scavenging activity was observed in the H3 (44.31 ± 0.97%) followed by H2, H0 and H1. H3 was significantly different to H0 and H1 ($p < 0.05$), while the other fractions did not show any significant difference from each other.

Among the HFB, HFP, HFT and HFN groups, the highest activities were found in HFB, which exhibited a greater potential than HFN peptides for eliminating hydroxyl radicals.

These results demonstrate that fractions containing smaller peptides have a higher antioxidant efficiency than the total hydrolysate. At the intestinal level, smaller bioactive peptides can cross the intestinal barrier without undergoing enzymatic hydrolysis and so can exhibit their biological activity in tissues. Another relevant factor is that the type of enzyme (animal, vegetable or microbial) used affects the antioxidant activity of peptides produced.

In the HFB group, H0, H2 and H3 did not influence the elimination activity of the

Table 1. Scavenging of the hydroxyl radical, DPPH and ABTS of the protein hydrolysates derived from buffalo milk casein and its fractions.

	HFB	HFP	HFT	HFN
		Hydroxyl (%)		
H0	34.84 ± 1.94 ^{Ab}	0.34 ± 4.00 ^{Bb}	0.00 ± 0.81 ^{Bb}	38.39 ± 1.47 ^{Ab}
H1	32.56 ± 2.95 ^{Ab}	0.00 ± 1.23 ^{Bb}	0.00 ± 3.51 ^{Bb}	37.37 ± 2.26 ^{Ab}
H2	44.07 ± 1.97 ^{Aa}	1.99 ± 0.96 ^{Cb}	5.59 ± 2.52 ^{Ba}	41.11 ± 2.16 ^{Aab}
H3	23.81 ± 3.02 ^{Bc}	5.54 ± 4.63 ^{Ca}	1.36 ± 1.32 ^{Db}	44.31 ± 0.97 ^{Aa}
		DPPH (%)		
H0	56.85 ± 2.13 ^{Aa}	56.93 ± 0.28 ^{Aa}	52.70 ± 0.35 ^{Bb}	45.83± 0.41 ^{Cc}
H1	50.97 ± 0.89 ^{Ab}	49.18 ± 0.70 ^{Ac}	51.27 ± 1.51 ^{Ab}	51.43 ± 0.73 ^{Ab}
H2	55.71 ± 0.78 ^{Aa}	52.54 ± 1.48 ^{Bb}	51.38 ± 1.07 ^{Bb}	51.32 ± 0.57 ^{Bb}
H3	54.71 ± 1.10 ^{Aa}	55.79 ± 0.37 ^{Aa}	55.22 ± 2.30 ^{Aa}	54.93 ± 0.48 ^{Aa}
		ABTS (%)		
H0	84.19 ± 1.36 ^{Aa}	84.02 ± 1.90 ^{Aa}	80.58 ± 1.98 ^{Aa}	70.04 ± 5.61 ^{Ba}
H1	6.97 ± 0.54 ^{Bc}	5.31 ± 0.91 ^{Bc}	5.27 ± 0.76 ^{Bb}	17.72 ± 1.59 ^{Ab}
H2	14.66 ± 1.59 ^{Ab}	12.15 ± 1.87 ^{Ac}	10.07 ± 0.40 ^{Ab}	12.96 ± 1.05 ^{Ab}
H3	77.43± 1.87 ^{Aa}	68.72 ± 3.26 ^{Bb}	74.54 ± 1.08 ^{ABa}	67.31 ± 5.16 ^{Ba}

Mean ± SE values with the same column superscripts (small alphabets) and in terms of line (upper alphabets) did not differ significantly (P <0.05). HFB: bubaline caseinate hydrolysate with bromelain (240 min); HFP: hydrolysed with papain (240 min); HFT: hydrolysed with trypsin (240 min); HFN: hydrolysed with neutrase (240 min) H0: hydrolysed integers; H1 with size (PM) in the range of> 10 kDa; H2: fraction with size (PM) in the range of 3-10 kDa and H3: fraction with size (PM) in the range of ≤ 3 kDa. Source: Authors.

DPPH radical, other than H1 (p> 0.05), however the radical removal capacity of these radicals was higher than H1. In the HFP group, the greatest activities were presented in the total hydrolysate and in the fraction <3 kDa (56.93 ± 0.28% and 55.79 ± 0.37%, respectively, which did not present significant differences among themselves (p <0. 05).

The hydrolysates obtained from trypsin and neutral protease digestion exhibited good radical elimination activities of 55.22 ± 2.30% and 54.93 ± 0.48%, respectively, by their H3 fractions. This was followed by H0, H1 and H2 for HFT and H1, H2 and H0 for HFN.

The variation in DPPH radical inhibition could be attributed to differences in enzyme specificity resulting in difference in the released functional peptides (Kumar, et al., 2016; Rabiei, et al., 2019; Tedesse & Emire, 2020). Similar results to the present DPPH scavenging activities were observed after treatment of whey protein isolates, smoothies and starch with

high hydrostatic pressure (Dong, et al., 2019; Andrés, Villanueva & Tenorio, 2016; Iskandar, et al., 2015).

The highest ABTS scavenging ability was exhibited by the H0 fractions of HTB (84.19 ± 1.36%) and HTN (70.04 ± 5.61%) and by the H3 fraction of HFB (77.43 ± 1.87%), which differed statistically ($p < 0.05$), while the lowest activities were found in the H1 and H2 fractions of HFB and HFN.

The hydrolysates and fractions produced by papain (84.02 ± 1.98%) and by trypsin (80.58 ± 1.90%) showed the highest ABTS cell turnover. The total hydrolysate had the highest potential for scavenging of the radical followed by the H3> H2> H1 fractions. The greatest potential for elimination of the total hydrolysate is due to the presence of peptides of different sizes that due to the synergistic effect, interaction between the peptides present in this fraction, potentiates the capture of the ABTS radical (Kumar, et al., 2016). In addition, the absence of antioxidant activity was determined by the ABTS free radical scavenging activity that is related to the type and time of enzymatic treatment (Hu, et al., 2017).

The highest iron chelation levels observed were over 70%, for the H3 (77.53 ± 0.26%), H2 (76.51 ± 0.11%), H3 (78.81 ± 2.16%) and H3 (84.76 ± 0.43%) fractions of bromelain, papain, trypsin and neutral protease digests, respectively (Table 2).

There were no significant differences copper chelation activities of the fraction from the HFB. In the HFT and HFN groups, the highest copper chelation activities were both found in H1 (64.35 ± 1.36% and 63.06 ± 3.93%, respectively) and the lowest in H0 (47.92 ± 0.84 and 38.23 ± 1.44, respectively), and these differed significantly from each other ($p < 0.05$).

Table 2. Chelation of iron and copper activity (%) of the protein hydrolysates derived from buffalo milk casein and its fractions.

	HFB	HFP	HFT	HFN
		Iron chelator (%)		
H0	76.31 ± 0.26 ^{Aa}	73.68 ± 2.35 ^{Ab}	75.15 ± 2.60 ^{Aa}	74.66 ± 0.63 ^{BCa}
H1	75.25 ± 0.69 ^{Aa}	76.30 ± 0.35 ^{Aab}	76.16 ± 0.20 ^{Aa}	75.94 ± 1.20 ^{Cab}
H2	76.64 ± 0.43 ^{Aa}	76.51 ± 0.11 ^{Aa}	76.14 ± 0.98 ^{Aab}	74.87 ± 1.09 ^{Ba}
H3	77.53 ± 0.26 ^{BCa}	75.70 ± 1.47 ^{Cab}	78.81 ± 2.16 ^{Ab}	84.76 ± 0.43 ^{Aa}
		Copper chelator (%)		
H0	66.62 ± 1.58 ^{Aa}	59.72 ± 2.33 ^{ABa}	47.92 ± 0.84 ^{Bcb}	38.23 ± 1.44 ^{Cb}
H1	56.18 ± 0.08 ^{Aa}	57.99 ± 2.68 ^{Aa}	64.35 ± 1.36 ^{Aa}	63.06 ± 3.93 ^{Aa}
H2	62.79 ± 1.13 ^{Aa}	60.75 ± 2.27 ^{Aa}	53.58 ± 0.79 ^{Aab}	61.64 ± 1.59 ^{Aa}
H3	64.18 ± 1.06 ^{Aa}	51.65 ± 0.38 ^{ABa}	55.75 ± 0.49 ^{ABab}	40.82 ± 0.08 ^{Bb}

Mean ± SE values with the same column superscripts (small alphabets) and in terms of line (upper alphabets) did not differ significantly (P <0.05). HFB: bubaline caseinate hydrolysate with bromelain (240 min); HFP: hydrolysed with papain (240 min); HFT: hydrolysed with trypsin (240 min); HFN: hydrolysed with neutrase (240 min) H0: hydrolysed integers; H1 with size (PM) in the range of> 10 kDa; H2: fraction with size (PM) in the range of 3-10 kDa and H3: fraction with size (PM) in the range of ≤ 3 kDa. Source: Authors.

Chelation of transition metals such as iron and copper, for example, can mitigate oxidative damage in the body and, in food, may act to decrease lipid peroxidation, thereby preventing changes in sensory characteristics (Sánchez-vioque, et al., 2013; Gulcin, 2020). The binding of protein hydrolysates to metal ions results in antioxidant effects (Wang, et al., 2020) since the peptides present in these hydrolysates bind to the transition metals that stop promoting oxidative damage, cell degeneration, breakage or oxidation of nucleic acids (Kurutas, 2016; Liu, et al., 2020).

Hydrolysates and fractions also exhibited inhibitory activity against α-glucosidase and α-amylase; However the inhibition of α-glucosidase in groups obtained by the action of bromelain, papain, trypsin and neutral protease (Table 3) were most significant in the H3 (51.51 ± 1.58%), H0 (51.51 ± 1.52%), H1; H2 (54.54 ± 0.00%) and H3 (27.27 ± 2.62) fractions, respectively, these differed statistically ($p < 0.05$). Inhibition of α-amylase was superior in the H2 fractions of all hydrolysates.

Table 3. Antidiabetic and antihypertensive activity (%) of the protein hydrolysates derived from buffalo milk casein and its fractions.

	HFB	HFP	HFT	HFN
		α-Glucosidase inhibitory (%)		
H0	36.36 ± 0.00 ^{Bb}	51.51 ± 1.52 ^{Aa}	42.42 ± 1.51 ^{ABa}	0.00 ± 0.00 ^{Cb}
H1	39.39 ± 1.52 ^{Bab}	24.24 ± 1.60 ^{Cb}	54.54 ± 0.00 ^{Aa}	0.00 ± 0.00 ^{Db}
H2	42.42 ± 1.51 ^{Aab}	9.09 ± 0.00^{Bc}	54.54 ± 0.00 ^{Aa}	0.00 ± 3.03 ^{Bb}
H3	51.51 ± 1.58 ^{Aa}	9.09 ± 0.00 ^{Bc}	45.45 ± 0.00 ^{Aa}	27.27 ± 2.62 ^{Ba}
		α-Amylase inhibitory (%)		
H0	73.58 ± 0.00 ^{Ab}	56.70 ± 0.77 ^{Bb}	44.74 ± 1.24 ^{Cd}	75.58 ± 0.24 ^{Aa}
H1	76.67 ± 0.00 ^{Aa}	53.33 ± 1.56 ^{Cc}	60.00 ± 1.14 ^{Bc}	75.44 ± 0.00 ^{Aa}
H2	75.44 ± 0.00 ^{Aab}	67.69 ± 0.43 ^{Ba}	68.42 ± 0.40 ^{Ba}	77.42 ± 0.00 ^{Aa}
H3	69.56 ± 0.00 ^{Bc}	39.13 ± 2.65 ^{Db}	65.85 ± 0.83 ^{Cb}	76.67 ± 0.00 ^{Aa}
		Inhibition of angiotensin-converting enzyme (ACE %)		
H0	62.66 ± 3.71^{Ab}	44.00 ± 0.14^{Bb}	39.35 ± 0.38^{Cc}	39.92 ± 0.57^{Cc}
H1	46.53 ± 4.99^{Ac}	40.82 ± 0.99^{Aa}	41.44 ± 0.00 ^{Ab}	41.25 ±0.47$_{Ab}$
H2	48.24 ± 1.94^{Ac}	42.25 ± 0.42^{Aa}	43.44 ± 0.00 ^{Ab}	42.49 ± 0.85^{Ab}
H3	80.23 ± 3.05^{Aa}	45.00 ± 1.14^{Ca}	61.08 ± 2.59^{Ba}	84.08 ± 1.97^{Aa}

Mean ± SE values with the same column superscripts (small alphabets) and in terms of line (upper alphabets) did not differ significantly (P <0.05). HFB: bubaline caseinate hydrolysate with bromelain (240 min); HFP: hydrolysed with papain (240 min); HFT: hydrolysed with trypsin (240 min); HFN: hydrolysed with neutrase (240 min) H0: hydrolysed integers; H1 with size (PM) in the range of> 10 kDa; H2: fraction with size (PM) in the range of 3-10 kDa and H3: fraction with size (PM) in the range of ≤ 3 kDa. Source: Authors.

The inhibition of α-amylase and α-glucosidase enzymatic activity leads to greater or lesser carbohydrate digestion and subsequent glycosidic absorption decreasing the availability of glucose in the bloodstream and resulting in the reduction of blood glucose in postprandial in diabetics (Jan, et al., 2016).

There have been several previous reports of results similar to the findings of the present study. Pino et al. (2020) reported that protein hydrolysates derived from whey can

inhibit α-glucosidase, while Kamal et al., (2018) reported inhibition of α-glucosidase from *Saccharomyces cerevisiae* by fractions of hydrolysates derived from bovine caseinate employing a plant protease.

The inhibitory potential against ACE was observed in all fractions and total hydrolysates. Inhibition activity of angiotensin-converting enzyme (ACE) I presented significant activity ($p < 0.05$) greater in fraction H3 (≤ 3 kDa) of all groups, assessed potential enzyme of 80.23 ± 3.05% for HFB, 45.00 ± 1.14% for HFP, 61.08 ± 2.59% to HFT and 84.08 ± 1.97% to HFN. In relation to the results obtained among the enzymatic treatments, bromelain was superior in relation to the other treatments.

Espejo-Carpio & Otte (2013) achieved between 30 and 65% inhibition of ACE by goat casein and whey hydrolysate produced using subtilisin and trypsin. Similar results were found by Luo et al. (2014), who found that a casein hydrolyse produced by three enzymes inhibited ACE by up to 65%.

The results found in this study are therefore comparable to the antihypertensive activities found in hydrolysates of casein obtained through enzymatic hydrolysis or by fermentation (Chaudhari, et al, 2017).

With respect to antimicrobial activity, the bromelain (HFB), papain (HFP), trypsin (HFT) and neutral protease (HFN) hydrolysates and fractions inhibited (Table 4) the growth of Gram-positive (*L. monocytogenes* and *S. aureus*) and negative bacteria (*E. coli* and *Salmonella* sp.).

Table 4. Antibacterial activity (%) of the protein hydrolysates derived from buffalo milk casein and its fractions.

	HFB	HFP	HFT	HFN
		Escherichia coli ATCC 25922		
H0	79.49 ± 2.42 ^{Aa}	45.50 ± 0.69 ^{Bab}	48.87 ± 4.37 ^{Bc}	45.07 ± 1.21 ^{Bc}
H1	67.52 ± 0.74 ^{Ab}	44.29 ± 0.52 ^{Cab}	59.92 ± 1.85 ^{BCb}	57.27 ± 0.43 ^{Bb}
H2	57.32 ± 1.60 ^{Bc}	41.05 ± 2.55 ^{Cb}	80.45 ± 0.12 ^{Aa}	78.24 ± 1.08 ^{Aa}
H3	47.32 ± 2.59 ^{Bd}	51.34 ± 7.76 ^{Bb}	83.77 ± 1.22 ^{Aa}	76.96 ± 6.76 ^{Aa}
		Listeria monocytogenes ATCC 19114		
H0	48.05 ± 2.90 ^{Bb}	62.22 ± 6.05 ^{Bc}	55.28 ± 4.04 ^{ABc}	43.44 ± 0.47 ^{Bc}
H1	52.38 ± 1.90 ^{Bb}	63.93 ± 0.95 ^{Ac}	61.60 ± 3.04 ^{ACB}	36.17 ± 0.80 ^{Ac}
H2	63.21 ± 6.05 ^{Ba}	75.43 ± 2.14 ^{Ab}	68.73 ± 1.71 ^{ABb}	26.95 ± 0.90 ^{Ab}
H3	62.93 ± 0.95 ^{Ba}	100.00 ± 4.94 ^{Aa}	92.73 ± 0.33 ^{Ac}	3.28 ± 0.43 ^{Ac}
		Salmonella typhimurium ATCC 14028		
H0	52.00 ± 1.90 ^{Bc}	49.38 ± 2.04 ^{Cc}	56.61 ± 1.19 ^{Ad}	69.30 ± 1.18 ^{Cd}
H1	54.52 ± 2.61 ^{Cb}	50.95 ± 1.14 ^{Dc}	66.44 ± 0.10 ^{Ac}	40.54 ± 0.43 ^{Bc}
H2	54.90 ± 1.85 ^{Cb}	53.75 ± 2.71 ^{Cb}	82.60 ± 0.00 ^{Ab}	20.96 ± 0.43 ^{Bb}
H3	63.12 ± 0.00 ^{Ca}	63.17 ± 1.28 ^{Ca}	99.29 ± 0.62 ^{Aa}	10.74 ± 0.48 ^{Ba}
		Staphylococcus aureus ATCC 25923		
H0	51.28 ± 1.76 ^{Ad}	46.72 ± 1.47 ^{ABd}	42.39 ± 1.71 ^{Bc}	55.75 ± 0.24 ^{Bc}
H1	60.55 ± 4.64 ^{Ac}	55.09 ± 0.33 ^{Bc}	47.43 ± 0.67 ^{Cc}	49.29 ± 0.43 ^{Bb}
H2	68.54 ± 3.76 ^{Bb}	91.87 ± 2.14 ^{Ab}	55.37 ± 0.52 ^{Cb}	49.57 ± 0.33 ^{Cb}
H3	100.00 ± 0.00 ^{Aa}	84.65 ± 5.81 ^{Ba}	75.00 ± 2.47 ^{Ca}	11.36 ± 0.62 ^{Bb}

Mean ± SE values with the same column superscripts (small alphabets) and in terms of line (upper alphabets) did not differ significantly (P <0.05). HFB: bubaline caseinate hydrolysate with bromelain (240 min); HFP: hydrolysed with papain (240 min); HFT: hydrolysed with trypsin (240 min); HFN: hydrolysed with neutrase (240 min) H0: hydrolysed integers; H1 with size (PM) in the range of> 10 kDa; H2: fraction with size (PM) in the range of 3-10 kDa and H3: fraction with size (PM) in the range of ≤ 3 kDa. Source: Authors.

E. coli is the most common contaminant in food (Kumar, et al., 2016). In this study, the highest antibacterial activity against this microorganism in the HFB group was exhibited by the total hydrolysate H0 (79.49 ± 2.42%), which differed from the other fractions (p <

0.05). The H3 fractions of HFP and HFT H3 exhibited the greatest ability to inhibit *E. coli*, while in the HFN group the H2 fraction was most effective (78.24 ± 1.08%), closely followed by H3 (76.96 ± 6.76%), both of which were significantly different from the other fractions ($p < 0.05$). In general, higher activities were observed in trypsin groups, being equal to HFB and HFN. Peptides with smaller molecular weights exhibit greater antibacterial activities than larger peptides (Abdel-Hamid, et al., 2016). Results similar to this study have been reported by Salami et al. (2010) using camel whey protein hydrolysates, which showed that a fraction of peptides under 3 kDa were most efficacious against *E. coli*.

Anti-listeria activity was greatest in the H3 fractions from bromelain (62.93 ± 0.95%), papain (100 ± 0.00%) and trypsin (92.73 ± 0.33%), which where were significantly ($p < 0.05$) greater that the other fractions. The neutral protease total hydrolysate (H0) exhibited the highest inhibitory capacity for *L. monocytogenes* compared with its fractions H1, H2 and H3. Comparison between the HFT and HFP exhibited greater potential of inhibition being statistically equal, followed by the HFB and HFN, which differ statistically between themselves.

Antimicrobial activity against *Salmonella* sp. was highest for the total casein hydrolysate produced by neutral protease (69.30 ± 1.18%), while the activities of the fractions decrease with peptide size. The H3 fraction of the bromelain, trypsin and papain hydrolysates exhibited significant inhibitory activity ($p < 0.05$). In general, the HFT group exhibited the highest inhibition potential.

Inhibition of *S. aureus* followed a trend similar to that found for *Salmonella* and *Lysteria*. The H3 fractions of the HFP and HFB groups were the same among themselves and differed from the other groups, with inhibitory activities of 100.00 ± 0.00%, 84.65 ± 5.81% and 75.00 ± 2.47%, respectively. In relation to the enzymatic groups, the group hydrolysed by bromelain had the highest average potential being equal to papain, followed by groups under the action of neutrase and trypsin, which presented lower significant potential ($p < 0.05$).

These results are consistent with those of Demers-Mathieu et al. (2013) who compared the ultrafiltered, filtered and total fractions from tryptic of hydrolysed bovine serum. Observing that the retained fraction of the ultrafiltration presented greater antimicrobial potential against Listeria sp. (49%), S. aureus (38%) and E. coli (97%) in relation to the total hydrolysate.

In this study, we observed greater antibacterial in filtered fractions compared to total hydrolysates, especially with respect to fraction H3. According to Osman et al. (2016), the presence of medium and small peptides can result in a greater antimicrobial activity; on the

other hand, a mixture of peptides of different weights (H0) can mean less antimicrobial activity when there is no synergism between the peptide fractions.

The presence or absence of antibacterial potential intensity mainly depends on the interaction between antimicrobial peptides and a bacterial cell wall, i.e., negatively charged peptides may exhibit greater affinity for positively charged membranes (Glinel, et al., 2012).

4 Conclusion

From the results presented in this study, it was concluded that the casein of hydrolysed buffalo milk can be fractionated using the ultrafiltration technique to obtain peptides of different molecular weight ranges to evaluate their biological activities and, consequently, their application in foods. Proteases such as bromelain, papain, trypsin and neutral protease are capable of hydrolyzing buffalo caseinate and producing bioactive peptides. In the present study, all fractions indicated the ability to chelate copper and iron, as well as antioxidant, antidiabetic and antimicrobial potential, especially the lower molecular with a higher biological potential compared to the other fractions. The presence of biological potential is due to the synergistic effect of the presence of peptides of different molecular weights present in the total hydrolysate and in their fractions the absence of this synergism, this characteristic is due to the way in which the peptide peptides interact with the radicals, with the enzymes or the bacterial cell wall. The results suggested that buffalo milk casein could be used as a natural source of dietary protein to obtain multifunctional bioactive peptides (antioxidant, antidiabetic and antibacterial activity). Encouraging the use of buffalo caseinate and derived peptides for direct human consumption and as a nutraceutical and pharmaceutical ingredient to improve its biofunctionality and increase shelf life.

Acknowledgments

This work was supported by grants from the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FACEPE (Fundação de Amparo à Ciência e Tecnologia de Pernambuco) – APQ-0460-2.01/15 and Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for financial support to the design.

References

Abd El-Salam, M. H., & El-Shibiny, S. (2017). Preparation, properties, and uses of enzymatic milk protein hydrolysates. *Critical Reviews in Food Science and Nutrition*, 57(6), 1119-1132. https://doi.org/10.1080/10408398.2014.899200

Abdel-Hamid, M., Goda, H. A., De Gobba, C., Jenssen, H., & Osman, A. (2016). Antibacterial activity of papain hydrolysed camel whey and its fractions. *International Dairy Journal*, 61, 91–98. https://doi.org/10.1016/j.idairyj.2016.04.004

Abdel-Hamid, M., Otte, J., De Gobba, C., Osman, A., & Hamad, E. (2017). Angiotensin I-converting enzyme inhibitory activity and antioxidant capacity of bioactive peptides derived from enzymatic hydrolysis of buffalo milk proteins. *International Dairy Journal*, 66, 91–98. https://doi.org/10.1016/j.idairyj.2016.11.006

Ali, H., Houghton, P. J., & Soumyanath, A. (2006). α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. *Journal of Ethnopharmacology*, 107(3), 449–455. https://doi.org/10.1016/j.jep.2006.04.004

Amigo, L., & Hernández-Ledesma, B. (2020). Current evidence on the bioavailability of food bioactive peptides. *Molecules*, 25(19), 4479. https://doi.org/10.3390/molecules25194479

Andrés, V., Villanueva, M. J., & Tenorio, M. D. (2016). The effect of high-pressure processing on colour, bioactive compounds, and antioxidant activity in smoothies during refrigerated storage. *Food Chemistry*, 192, 328–335. https://doi.org/10.1016/j.foodchem.2015.07.031

Bamdad, F., Bark, S., Kwon, C. H., Suh, J. W., & Sunwoo, H. (2017). Anti-inflammatory and antioxidant properties of peptides released from β-lactoglobulin by high hydrostatic pressure-assisted enzymatic hydrolysis. *Molecules*, 22(6), 949. https://doi.org/10.3390/molecules22060949

Chaudhari, D. D., Singh, R., Mallappa, R. H., Rokana, N., Kaushik, J. K., Bajaj, R., Batish, V. K., & Grover, S. (2017). Evaluation of casein & whey protein hydrolysates as well as milk

fermentates from *Lactobacillus helveticus* for expression of gut hormones. *The Indian Journal of Medical Research*, 146(3), 409–419. https://doi.org/10.4103/ijmr.IJMR_802_15

Cumby, N., Zhong, Y., Naczk, M., & Shahidi, F. (2008). Antioxidant activity and water-holding capacity of canola protein hydrolysates. *Food Chemistry*, 109(1), 144–148. https://doi.org/10.1016/j.foodchem.2007.12.039

Demers-Mathieu, V., Gauthier, S. F., Britten, M., Fliss, I., Robitaille, G., & Jean, J. (2013). Antibacterial activity of peptides extracted from tryptic hydrolysate of whey protein by nanofiltration. *International Dairy Journal*, 28(2), 94–101. https://doi.org/10.1016/j.idairyj.2012.09.003

Dong, X. H., Li, J., Jiang, G. X., Li, H. Y., Zhao, M. M., & Jiang, Y. M. (2019). Effects of combined high pressure and enzymatic treatments on physicochemical and antioxidant properties of peanut proteins. *Food Science & Nutrition*, 7(4), 1417-1425. https://doi.org/10.1002/fsn3.976

Duan, X. J., Zhang, W. W., Li, X. M., & Wang, B. G. (2006). Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*. *Food Chemistry*, 95(1), 37–43. https://doi.org/10.1016/j.foodchem.2004.12.015

Egito, A. S., Girardet, J. M., Laguna, L. E., Poirson, C., Mollé, D., Miclo, L., … Gaillard, J. L. (2007). Milk-clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine κ-casein. *International Dairy Journal*, 17(7), 816–825. https://doi.org/10.1016/j.idairyj.2006.09.012

Espejo-Carpio, F. J., De Gobba, C., Guadix, A., Guadix, E. M., & Otte, J. (2013). Angiotensin I-converting enzyme inhibitory activity of enzymatic hydrolysates of goat milk protein fractions. *International Dairy Journal*, 32(2), 175–183. https://doi.org/10.1016/j.idairyj.2013.04.002

Fangmeier, M., Kemerich, G. T., Machado, B. L., Maciel, M. J., Souza, C. F. V. D. (2019). Effects of cow, goat, and buffalo milk on the characteristics of cream cheese with whey retention. *Food Science and Technology*, 39, 122-128. https://doi.org/10.1590/fst.39317

Fosgerau, K., & Hoffmann, T. (2015). Peptide therapeutics: Current status and future directions. *Drug Discovery Today*, 20(1), 122–128. ttps://doi.org/10.1016/j.drudis.2014.10.003

Gaudel, C., Nongonierma, A. B., Maher, S., Flynn, S., Krause, M., Murray, B. A., Newsholme, P. (2013). A Whey protein hydrolysate promotes insulinotropic activity in a clonal pancreatic -cell line and enhances glycemic function in ob/ob mice. *Journal of Nutrition*, 143(7), 1109–1114. https://doi.org/10.3945/jn.113.174912

Glinel, K., Thebault, P., Humblot, V., Pradier, C. M., & Jouenne, T. (2012). Antibacterial surfaces developed from bio-inspired approaches. *Acta Biomaterialia*, 8(5), 1670–1684. https://doi.org/10.1016/j.actbio.2012.01.011

Gulcin, İ. (2020). Antioxidants and antioxidant methods: an updated overview. *Archives of Toxicology*, 94(3), 651-715. https://doi.org/10.1007/s00204-020-02689-3

Hernández-Ledesma, B., Quirós, A., Amigo, L., & Recio, I. (2007). Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. *International Dairy Journal*, 17(1), 42–49. https://doi.org/10.1016/j.idairyj.2005.12.012

Hu, G., Zheng, Y., Liu, Z., Xiao, Y., Deng, Y., & Zhao, Y. (2017). Effects of high hydrostatic pressure, ultraviolet light-C, and far-infrared treatments on the digestibility, antioxidant and antihypertensive activity of α-casein. *Food Chemistry*, 221, 1860–1866. https://doi.org/10.1016/j.foodchem.2016.10.088

Ingham, B., Smialowska, A., Kirby, N. M., Wang, C., & Carr, A. J. (2018). A structural comparison of casein micelles in cow, goat and sheep milk using X-ray scattering. *Soft Matter*, 14(17), 3336-3343. https://doi.org/10.1039/C8SM00458G

Iskandar, M., Lands, L., Sabally, K., Azadi, B., Meehan, B., Mawji, N., Kubow, S. (2015). High hydrostatic pressure pretreatment of whey protein isolates improves their digestibility and antioxidant capacity. *Foods*, 4(2), 184–207. https://doi.org/10.3390/foods4020184

Islam, M. A., Alam, M. K., Islam, M. N., Khan, M. A. S., Ekeberg, D., Rukke, E. O., & Vegarud, G. E. (2014). Principal milk components in buffalo, holstein cross, indigenous cattle and red chittagong cattle from Bangladesh. *Asian-Australasian Journal of Animal Sciences*, 27(6), 886–897. https://doi.org/10.5713/ajas.2013.13586

Jan, F., Kumar, S., & Jha, R. (2016). Effect of boiling on the antidiabetic property of enzyme treated sheep milk casein. *Veterinary World*, 9(10), 1152–1156. https://doi.org/10.14202/vetworld.2016.1152-1156

Kamal, H., Jafar, S., Mudgil, P., Murali, C., Amin, A., & Maqsood, S. (2018). Inhibitory properties of camel whey protein hydrolysates toward liver cancer cells, dipeptidyl peptidase-IV, and inflammation. *Journal of Dairy Science*, 101(10), 8711-8720. https://doi.org/10.3168/jds.2018-14586

Kim, Y. M., Wang, M. H., & Rhee, H. I. (2004). A novel α-glucosidase inhibitor from pine bark. *Carbohydrate Research*, 339(3), 715–717. https://doi.org/10.1016/j.carres.2003.11.005

Konrad, B., Anna, D., Marek, S., Marta, P., Aleksandra, Z., & Józefa, C. (2014). The evaluation of dipeptidyl peptidase (DPP)-IV, α-glucosidase and angiotensin converting enzyme (ACE) inhibitory activities of whey proteins hydrolysed with serine protease isolated from asian pumpkin (*Cucurbita ficifolia*). *International Journal of Peptide Research and Therapeutics*, 20(4), 483–491. https://doi.org/10.1007/s10989-014-9413-0

Kumar, D., Chatli, M. K., Singh, R., Mehta, N., & Kumar, P. (2016). Antioxidant and antimicrobial activity of camel milk casein hydrolysates and its fractions. *Small Ruminant Research*, 139, 20–25. https://doi.org/10.1016/j.smallrumres.2016.05.002

Kurutas, E. B. (2016). The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. *Nutrition Journal*, 15(71), 1-22. https://doi.org/10.1186/s12937-016-0186-5

Liu, Z., Chen, X., Li, Z., Ye, W., Ding, H., Li, P., & Aung, L. H. H. (2020). Role of RNA oxidation in neurodegenerative diseases. *International Journal of Molecular Sciences*, 21(14), 5022. https://doi.org/10.3390/ijms21145022

Luo, Y., Pan, K., & Zhong, Q. (2014). Physical, chemical and biochemical properties of casein hydrolysed by three proteases: Partial characterizations. *Food Chemistry*, 155, 146–154. https://doi.org/10.1016/j.foodchem.2014.01.048

Megías, C., Pedroche, J., Yust, M. M., Julio Girón-Calle, M. A., Millán, F., & Vioque, J. (2007). Affinity purification of copper chelating peptides from chickpea protein hydrolysates. *Journal of Agricultural and Food Chemistry*, 55(10), 3949–3954. https://doi.org/10.1021/jf063401s

Muguerza, B., Ramos, M., Sánchez, E., Manso, M. A., Miguel, M., Aleixandre, A., … Recio, I. (2006). Antihypertensive activity of milk fermented by *Enterococcus faecalis* strains isolated from raw milk. *International Dairy Journal*, 16(1), 61–69. https://doi.org/10.1016/j.idairyj.2005.01.001

Nielsen, S. D., Beverly, R. L., Qu, Y., & Dallas, D. C. (2017). Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chemistry*, 232, 673–682. https://doi.org/10.1016/j.foodchem.2017.04.056

Osman, A., Goda, H. A., Abdel-Hamid, M., Badran, S. M., & Otte, J. (2016). Antibacterial peptides generated by Alcalase hydrolysis of goat whey. *LWT - Food Science and Technology*, 65, 480–486. https://doi.org/10.1016/j.lwt.2015.08.043

Petsantad, P., Sangtanoo, P., Srimongkol, P., Saisavoey, T., Reamtong, O., Chaitanawisuti, N., & Karnchanatat, A. (2020). The antioxidant potential of peptides obtained from the spotted babylon snail (*Babylonia areolata*) in treating human colon adenocarcinoma (Caco-2) cells. *RSC Advances*, 10(43), 25746-25757. https://doi.org/10.1039/D0RA03261A

Pietrzak-Fiećko, R., & Kamelska-Sadowska, A. M. (2020). The comparison of nutritional value of human milk with other mammals' milk. *Nutrients*, 12(5), 1404. https://doi.org/10.3390/nu12051404

Rabiei, S., Rezaei, M., Asgharzade, S., Nikoo, M., & Rafieia-kopai, M. (2019). Antioxidant and cytotoxic properties of protein hydrolysates obtained from enzymatic hydrolysis of Klunzinger's mullet (*Liza klunzingeri*) muscle. *Brazilian Journal of Pharmaceutical Sciences*, 55. https://doi.org/10.1590/s2175-97902019000218304

Rivero-Pino, F., Espejo-Carpio, F. J., & Guadix, E. M. (2020). Antidiabetic food-derived peptides for functional feeding: Production, functionality and in vivo evidences. *Foods*, 9(8), 983. https://doi.org/10.3390/foods9080983

Saiga, A., Tanabe, S., & Nishimura, T. (2003). Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. *Journal of Agricultural and Food Chemistry*, 51(12), 3661–3667. https://doi.org/10.1021/jf021156g

Salami, M., Moosavi-Movahedi, A. A., Ehsani, M. R., Yousefi, R., Haertlé, T., Chobert, J. M., Niasari-Naslaji, A. (2010). Improvement of the antimicrobial and antioxidant activities of camel and bovine whey proteins by limited proteolysis. *Journal of Agricultural and Food Chemistry*, 58(6), 3297–3302. https://doi.org/10.1021/jf9033283

Sánchez-vioque, R., Polissiou, M., Astraka, K., & Mozos-Pascual, M. D. L. (2013). Polyphenol composition and antioxidant and metal chelating activities of the solid residues from the essential oil industry. *Food Chemistry*, 49(141), 150–159. https://doi.org/10.1016/j.indcrop.2013.04.053

Sánchez-Vioque, R., Rodríguez-Conde, M. F., Reina-Ureña, J. V., Escolano-Tercero, M. A., Herraiz-Peñalver, D., & Santana-Méridas, O. (2012). In vitro antioxidant and metal chelating properties of corm, tepal and leaf from saffron (*Crocus sativus* L.). *Industrial Crops and Products*, 39(1), 149–153. https://doi.org/10.1016/j.indcrop.2012.02.028

Sawicka, B., Ziarati, P., Krochmal-Marczak, B. A. R. B. A. R. A., Skiba, D. (2020). Nutraceuticals in food and pharmacy. A Review. *Annales UMCS Sectio e Agricultura*, 74(4), 7-30. http://dx.doi.org/10.24326/as.2019.4.2

Shanmugam, V. P., Kapila, S., Sonfack, T. K., & Kapila, R. (2015). Antioxidative peptide derived from enzymatic digestion of buffalo casein. *International Dairy Journal*, 42, 1–5. https://doi.org/10.1016/j.idairyj.2014.11.001

Shazly, A. B., He, Z., El-Aziz, M. A., Zeng, M., Zhang, S., Qin, F., & Chen, J. (2017). Fractionation and identification of novel antioxidant peptides from buffalo and bovine casein hydrolysates. *Food Chemistry*, 232, 753–762. https://doi.org/10.1016/j.foodchem.2017.04.071

Shazly, A. B., Mu, H., Liu, Z., Abd El-Aziz, M., Zeng, M., Qin, F., Chen, J. (2019). Release of antioxidant peptides from buffalo and bovine caseins: Influence of proteases on antioxidant capacities. *Food Chemistry*, 274, 261-267. https://doi.org/10.1016/j.foodchem.2018.08.137

Sujarwanta, R.O. et al. (2018). Increased inhibition of angiotensin converting enzyme (ACE) obtained from Indonesian buffalo meat protein using SEP-PAK Plus C18. *Pakistan Journal of Nutrition*, 17(9): 434 -440. https://doi.org/10.3923/pjn.2018.434.440

Tadesse, S. A., & Emire, S. A. (2020). Production and processing of antioxidant bioactive peptides: A driving force for the functional food market. *Heliyon*, 6(8), e04765. https://doi.org/10.1016/j.heliyon.2020.e04765

Trommelen, J., Weijzen, M. E., van Kranenburg, J., Ganzevles, R. A., Beelen, M., Verdijk, L. B., & van Loon, L. J. (2020). Casein protein processing strongly modulates post-prandial plasma amino acid responses in vivo in humans. *Nutrients*, 12(8), 2299. https://doi.org/ 10.3390/nu12082299

Wang, P. et al. (2020). Preparation of antioxidant peptides from hairtail surimi using hydrolysis and evaluation of its antioxidant stability. *Food Science and Technology*, 40(4), 945-955. https://doi.org/10.1590/fst.23719

Wu, S., Qi, W., Li, T., Lu, D., Su, R., & He, Z. (2013). Simultaneous production of multi-functional peptides by pancreatic hydrolysis of bovine casein in an enzymatic membrane reactor via combinational chromatography. *Food Chemistry*, 141(3), 2944–2951. https://doi.org/10.1016/j.foodchem.2013.05.050

Zarei, M., Ebrahimpour, A., Abdul-Hamid, A., Anwar, F., Bakar, F. A., Philip, R., & Saari, N. (2014). Identification and characterization of papain-generated antioxidant peptides from palm kernel cake proteins. *Food Research International*, 62, 726–734. https://doi.org/10.1016/j.foodres.2014.04.041

Zhang, C., Cao, W., Hong, P., Ji, H., Qin, X., & He, J. (2009). Angiotensin I-converting enzyme inhibitory activity of *Acetes chinensis* peptic hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. *International Journal of Food Science and Technology*, 44(10), 2042–2048. https://doi.org/10.1111/j.1365-2621.2009.02028.x

Zhao, Q., Shi, Y., Wang, X., & Huang, A. (2020). Characterization of a novel antimicrobial peptide from buffalo casein hydrolysate based on live bacteria adsorption. *Journal of Dairy Science*, 103(12). https://doi.org/10.3168/jds.2020-18577

Percentage of contribution of each author in the manuscript

Wellington Leal dos Santos – 35%

Thailan Arlindo da Silva – 15%

Patrícia Lins Azevedo do Nascimento – 10%

Rosângela Estevão Alves Falcão – 10%

João Tiago Correia Oliveira – 15%

Keila Aparecida Moreira – 15%