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Research Article

Bacillus subtilis: A Probiotic Promiser in Yogurt Production with Antioxidant Potential

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Abstract

The demand for natural and functional foods has increased in recent decades. Probiotic bio-yogurts whose microbiota can benefit the organism by participating in maintaining the balance of the human microbiota, reducing the effects of oxidative stress and increasing the shelf life of food. The objective was to produce bio-yogurt with and without lactose with addition of *Bacillus subtilis* and to evaluate the antioxidant potential of the peptides released during the fermentation process and its fractions. For bio-yogurt production a simple factorial arrangement was used in evaluating two types of milk (lactose and lactose-free), adding probiotic culture containing *Bacillus subtilis*. The obtained peptide extract was subjected to ultrafiltration with cut membranes and the fractions evaluated in relation to the antioxidant potential of scavenging of the ABTS radical, DPPH, hydroxyl, chelating copper and iron. The pH of the bio-yogurts was approximately 4. Good water retention capacity and relatively low carbon consumption were observed. In the evaluation of the results, the peptides obtained from the different combinations of milk and probiotic culture presented antioxidant potential. In addition, the results show that *Bacillus subtilis* is a promising probiotic in the production of bio-yogurt with antioxidant potential increasing its shelf life. Moreover, the peptides obtained can be used as nutraceutical ingredients in functional foods.

Keywords: Antioxidant Activity; Bacillus subtilis; Bio-Yogurt; Functional Food; Probiotic.

Abbreviations

WHC: Water Holding Capacity; DNS: 3,5-Dinitrosalicylic Acid; UHT: Ultra High Temperature; ABTS: 2,2'-azinobis-3-ethylbenzthiazoline-6-sulfonic Acid; DPPH: 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic Acid; CA: Chelating Activity; HRSA: Hydroxyl Radical Scavenging Activity; SRS: Superoxide Radical Scavenging; LACT: Lactose; PC: Probiotic Culture; CV: Coefficient of Variation ; TP: Total Peptides.

Introduction

Food protein is of great importance for human and animal nutrition [1,2]. When ingested, it undergoes structural changes resulting in the release and absorption of essential amino acids and peptides to meet our nutritional needs [3]. Peptides are protein fragments formed from the union of two or more amino acids through peptide bonds [4]. Many of these compounds may present chemical structures similar to the endogenous peptides and take on biological activities such as antihypertensive, antimicrobial, anticoagulant, antidiabetic, neuroprotective, antioxidant actions, among others [5,6].

Antioxidants act by scavenging free radicals from both oxidative metabolism in living organisms and lipid peroxidation in food [7]. These biomolecules play major roles in the most diverse systems preventing the installation of pathological processes like carcinogenesis, neurodegeneration, diabetes, cardiovascular diseases, etc [8].

Several reactive oxygen species, including free radicals such as superoxide anion (O_2 •), hydroxyl radicals (•HO) and non-free radicals, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), may be responsible for these harmful reactions and are widely researched when testing the scavenging capacity of these radicals by the action of antioxidant substances [9].

Among the numerous known antioxidant substances, antioxidant peptides can be produced from the action of digestive enzymes and microbial fermentation [1,2]. Functional foods, especially fermented probiotics such as bio-yogurt, stand out in this context as they are widely studied and marketed by the nutraceutical and food industries [10].

Although the major importance of probiotics is in maintaining the balance of the human microbiota, the microorganisms added to the fermented foods are able to hydrolyze the food protein and produce peptides with antioxidant activities [11,12]. Thus, the demand for and consumption of natural and functional foods that bring benefits to the organism, such as probiotic yogurts, has increased, justified by the fact that the current concern leads to improvement in quality of life [13].

This study sought to produce bio-yogurt with and without lactose with addition of *Bacillus subtilis* and evaluate the antioxidant potential of the peptides released during the fermentation process as well as its fractions.

Materials and Methods

Bio-yogurt preparation

Pasteurized UHT (ultra high temperature) lactose and lactose-free bovine milk underwent an additional heat treatment (91 ± 1°C/10 min). The milk was cooled to 45°C and the lyophilized starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were inoculated at the concentration of 0.6 g.L-1. The probiotic culture constituted of *Bacillus subtilis* (0.25 $g.L^{-1}$). Fermentation was carried out at 45 ± 1°C for a period of 12 hours (Table 1) and the yogurt fermentation end point was based on clot tightness check. Subsequently, the product was cooled to 4 ± 1°C and the clot was broken by manual shaking with a glass rod.

Determination of pH

To determine the pH value of the sample, 10 mL of the bio-yogurts was added to tubes and measured using digital pH meter [14]. The readings were performed in triplicates.

Determination of water holding capacity

The water holding capacity (WHC) was determined in triplicate according to the modified method of Parnell-Clunies., *et al.* [15]. 20g of bio-yogurt was centrifuged at 3400 g for 10 minutes at 10°C. After centrifugation, the supernatant was drained and weighed. The capacity to receive water was published in percentage (%), according to the following equation (Eq. 1):

$$WHC = \left(\frac{\text{sample mass} - \text{mass of the supernatant}}{\text{sample mass}} \right) * 100 \qquad (1)$$

Determination of reducing sugars

The dosage of glucose and fructose during fermentation for the production of bio-yogurt was carried out by the determination of reducing sugars adapted from that described by Santos., *et al.* [16]. The reaction was run on a 96-well polystyrene microplates (Corning®, Tewksbury, MA) where 25µL of sample and 25µL of the DNS (3,5-dinitrosalicylic acid) reagent were added. Calibration curves were prepared using five concentrations of glucose and fructose (0.2, 0.4, 0.8, 1.6 and 2.0 $g.L^{-1}$). The microplates were sealed and boiled for 5 minutes. They were then cooled on ice, and 330 µL of water were added. Samples were read at 490 nm. The concentrations, in gL^{-1} of glucose or fructose, were calculated based on the line-of-best-fit generated from the calibration curve.

Obtaining the crude extract of the peptide and its fractions

The bio-yogurt samples were centrifuged at 5000 g at 4°C for 30 minutes. Subsequently, the supernatants were ultrafiltered sequentially through an ultrafiltration unit Amicon® with different molecular weights, for 60 minutes at 4°C and centrifuged at 4000 g, according to methods described by Kumar., *et al.* [17]. The total peptides (TP) were ultrafiltered through a 10 kDa membrane, yielding two fractions; retained (> 10 kDa) and permeate (≤10 kDa). The permeate was ultrafiltered through a 3 kDa membrane to obtain the second retentate (3 ≥ n ≤ 10 kDa; 3-10 kDa) and permeate (≤ 3 kDa; 3 kDa).

ABTS Radical Cation Assay

The antioxidant activity assay involving the removal of the cation radical ABTS+•, generated from the oxidation of 7 mM 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) with 2.45 mM potassium persulfate, -incubated in the dark for 12 hours before use. The ABTS+• solution was adjusted to absorbance of 0.700 ± 0.02 at 734 nm in a spectrophotometer. It was dilution in 5 mM phosphate buffer, and performed according to methods described by Hernández-Ledesma., *et al.* [18]. A 50 µL aliquot of the sample was mixed with 950 µL of the diluted ABTS+• solution, the reaction mixture was incubated for 10 minutes in the dark at room temperature (25 °C). The absorbance of the reaction was measured at 734 nm and the scavenging activity of the ABTS+• radical was calculated according to the equation (Eq. 2):

$$ABTS\ (\%) = \left[\left(\frac{A_{control} - A_{sample}}{A_{control}} \right) \right] * 10 \qquad (2)$$

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

DPPH Free Radical Scavenging Assay

The activity of scavenging of the radical DPPH• (2,2-diphenyl-1,4-phenylenediamine) was determined according to the methods described by Yen and Glen [19]. The reaction mixture consists

of 200 µL of the sample and 200 µL of ethanolic solution of DPPH• 0.16 mM in 96-well polystyrene microplates (Corning®, Tewksbury, MA). The reaction was incubated for 30 minutes in the dark, and the absorbance was measured at 517 nm. The scavenging capacity of the DPPH• radical was calculated using the equation (Eq. 3):

$$DPPH(\%) = \left[1 - \left(\frac{A_{sample} - A_{\text{white}}}{A_{control}}\right)\right] * 100 \quad (3)$$

Where the A_{sample} is the absorbance of the samples, A_{white} is the absorbance of the sample without DPPH• and $A_{controle}$ is the absorbance of the control (DPPH solution).

Chelating activity of $Fe2^{+}$ and $Cu2^{+}$

The iron chelating activity (CA) was performed with modifications, using 125 µL of the samples, mixed with 0.5 mL of sodium acetate buffer (0.1 M, pH 4.9) and 12.5 µL of Fe^{2+} (2 mM). This mixture was incubated for 30 minutes and then 50 µL of the ferrozine solution (5 mM) was added after a further 30 minutes incubation. The absorbance was measured at 562 nm [20].

Copper chelating activity was performed with modifications employing 0.5 mL of sodium acetate buffer (pH 6.0, 50 mM) mixed with 12.5 µL $CuSO_4$ solution (5 mM) and 125 µL of the samples. This mixture was incubated for 30 minutes and then 12.5 µL of the pyrocatechol violet solution (4 mM) was added after a further 30 minutes incubation. The absorbance of the reaction mixture was read at 632 nm using a spectrophotometer [21].

For both activities, the negative control ($A_{control}$) was performed using water in substitution for the sample.

The positive control was performed with 0.045% EDTA solution and the percentage of inhibition was determined according to the equation (Eq. 4) below:

$$CA\ (\%) = \left[\frac{(A_{control} - A_{sample})}{A_{control}}\right] * 100 \quad (4)$$

Hydroxyl radical scavenging activity

The tests for the determination of hydroxyl radical scavenging (HRSA) were determined according to methods described by Zhu., *et al*. [22]. Were modified it by using 30 µL of the sample, 50 µL of 1,10-phenanthroline (5.0 mM), 50 µL of $FeSO_4$ (5.0 mM), 50 µL of EDTA (15 mM) and 30 µL of sodium phosphate buffer, 2 M, pH 7.4) in 96 well polystyrene microplates (Corning®, Tewksbury, MA). After 5 minutes, 60 µL of H_2O_2 was added to initiate the scavenging reaction. The reaction mixture was incubated for 1 h at 37°C and absorbance was measured using a microplate reader. The hydroxyl radical removal activity was calculated according to the equation (Eq. 5) below;

$$HRSA = \left(\frac{A_{sample} - A_{\text{degraded}}}{A_{\text{non-degraded}} - A_{\text{degraded}}}\right) * 100 \quad (5)$$

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.

Superoxide radical scavenging activity

The superoxide radical scavenging (SRS) activity was determined according to the method described by Bombad., *et al.* [23]. This was modified by using 80 µL of the samples were mixed in 80 µL of 50 mM Tris-HCl-EDTA buffer (1 mM, pH 8.2). The reaction mixture was incubated at 25 °C for 10 minutes. Subsequently, 40 µL of 1.5 mM pyrogallol solution was added and Tris-HCl-EDTA buffer was used as the control. The absorbance was determined at 340 nm at time zero and after 5 minutes. The scavenging capacity of the superoxide radical was quantified according to equation (Eq. 6):

$$SRS(\%) = \left[\frac{\Delta A_{control} - \Delta_{sample}}{\Delta A_{control}}\right] * 100 \quad (6)$$

Where, $A_{control}$ represents the absorbance per minute of the control solution containing pyrogallol and buffer, and A_{sample} represents the absorbance per minute of the sample.

Statistical analysis

The parameters were analyzed in triplicate and the data expressed as means and standard deviations. The experimental design was a factorial scheme (2 x 2), totaling 4 trials. Significant differences between the bio-yogurts submitted to different additions of probiotic culture were obtained using unidirectional analysis of variance followed by evaluation of differences between the means using the multiple comparison test of Tukey in SISVAR version 5.6.

Results and Discussion

The pH of the bio-yogurt samples remained between 3.75 and 4.36. The sample of bio-yogurt without lactose and without addition of *Bacillus subtilis* presented higher pH value ($p<0,05$), differing from the others statistically. The lowest value was observed in system 4 (milk with lactose and presence of *B. subtilis*), which shows lactose and probiotic culture (Table 1). Studies by Parnell-Clunies., *et al.* [15] demonstrate a strong correlation between the pH values of the fermentative systems and the production of lactic acid. The latter is a result of the microbial fermentation. It was observed that the systems that presented higher acidification were those with higher nutrient complexity, especially lactose, which, when fermented, produces lactic acid which causes a decrease in pH.

	LACT	PCC	WHC (%)	Glucose (mg.L^{-1})	Fructose (mg.L^{-1})	pH
1	Absence	Absence	65.21 c	13.74 c	8.13 bc	4.36 a
2	Presence	Absence	64.14 a	30.98 a	18.26 a	3.89 b
3	Absence	Presence	64.59 b	9.58 d	5.72 c	3.91 b
4	Presence	Presence	64.33 a	25.24 b	14.95 ab	3.75 c
	CV (%)		0.25	3.30	2.95	0.28

Table 1: Water retention capacity, reducing sugars and pH of lactose-free yogurt and lactose using *Bacillus subtilis* as probiotic culture.

* LACT- Lactose; PCC – Probiotic culture containing *Bacillus subtilis*; WHC- Water holding capacity; Different letters in the same column differ statistically from one another $p < 0.05$.

Regarding water retention capacity (WHC) greater than 60% in all systems, these values were very similar to those found by Akalin., *et al.* [24] which ranged from 50 to 70% when testing a probiotic bio-yogurt fortified with sodium and calcium caseinate and concentrated protein.

Reducing sugars differed statistically among all treatments, presenting higher values in systems containing lactose without addition of *Bacillus* sp. (30.98 and 18.26%, glucose and fructose, respectively). Treatments containing lactose and probiotic culture recorded 25.24 and 14.95% glucose and fructose, respectively.

Glucose and fructose play an important role in the fermentation processes. The detection of these monosaccharides helps in the analysis of the speed of consumption of the carbon sources and how much is no longer fermented [16]. In the present study, the consumption of hexoses was relatively low, demonstrating that the production of bio-yogurts under the analyzed conditions can be efficient [25].

The antioxidant activity of scavenging of the ABTS+ radical (Table 2) was higher in the total fraction, followed by the fraction 3 kDa and of 3-10 kDa for all treatments differing statistically ($p<0.05$). Regarding the scavenging activity of the DPPH radical, it was observed in higher amounts in the 3kDa fraction and lower in the total fraction. Both activity systems containing lactose differ in some trials from those where lactose is absent, the same occurs in systems containing *Bacillus subtilis.*

ABTS (%)					
System	Lactose	Probiotic	TP*	10-3 kDa	3 kDa
01	Absence	Absence	91.33 aA	45.52 aC	80.42 aB
02	Presence	Absence	79.54 bB	42.51 aC	67.19 bB
03	Absence	Presence	90.32 aA	43.97 aC	80.63 aB
04	Presence	Presence	80.58 bA	38.67 aC	67.09 bB
CV (%) = 4.91					
DPPH (%)					
01	Absence	Absence	24.28 bC	54.67 aB	93.38 aA
02	Presence	Absence	36.67 cB	40.90 bB	76.06 bA
03	Absence	Presence	12.29 aC	60.35 aB	93.88 aA
04	Presence	Presence	27.93 bC	43.49 bB	76.23 bA
CV (%) = 5.61					

Table 2: Antioxidant activity of scavenging of the ABTS and DPPH radicals of the added bio-yogurt of probiotic culture.

*TP – Total peptides derived from bio-yogurt; CV - Coefficient of variation; Different lowercase letters on the same line differ from each other in relation to the lactose-probiotic system; Different capital letters on the same line results differ statistically in relation to the weight of the peptides.

In the present study, it is possible to observe a synergistic effect [26], that is, the presence of peptides with different sizes can lead to a higher antioxidant potential (ABTS radical removal). However, the mixture of peptides of different molecular weights may mean less potential biological activity in the absence of synergism [27], as observed in the DPPH radical scavenging activity.

The ability to chelate metal ions was evaluated (Table 3) and it was observed that the ability to chelate iron is higher in the peptides of lower molar weight differing from the others. Similar results were observed in the chelation of copper in which the peptides present in the total and lower weight fractions presented higher chelation potential and were statistically equal ($p < 0.05$). Furthermore, the 3-10 kDa fraction showed no chelation ability to metal ions.

The chelation of metal ions acts on the body in the reduction of lipid peroxidation which avoids modifications in the flavor of food [28]. In addition, when absorbed, the peptides that have these characteristics promote a decrease of the oxidative damages [29].

The activities of scavenging of the hydroxyl radicals and superoxide that are little studied due to the difficulty to obtain organic molecules capable of binding to these radicals presented encouraging results (Table 4). The ability to scavenge the hydroxyl had maximum potential in almost all the tests, not differing among them, except among the weights of system 2. In relation to the ability to sequester the superoxide radical, a greater anti-superoxide potential was observed in the fraction 3-10 kDa in systems 2 (presence lactose and absence of *Bacillus subtilis*) and 3 (absence lactose and presence of *B. subtilis*) that differ between peptide sizes but are equal to each other.

Iron chelating (%)					
System	Lactose	Probiotic	TP*	10-3 kDa	3 kDa
01	Absence	Absence	2.83 abB	0.00 aB	22.11 aA
02	Presence	Absence	1.75 bB	0.00 aB	22.53 aA
03	Absence	Presence	5.99 aB	0.00 aC	15.88 bA
04	Presence	Presence	4.67 abB	0.00 aC	14.84 bA
CV (%) = 4.81					
Copper chelating (%)					
01	Absence	Absence	84.08 aA	0.00 aB	84.04 abA
02	Presence	Absence	85.30 aA	0.00 aB	89.12 aA
03	Absence	Presence	82.14 aA	0.00 aB	79.49 bA
04	Presence	Presence	68.06 bB	0.00 aC	88.82 aA
CV (%) = 4.54					

Table 3: Ability to chelating iron and copper of the water-soluble peptides of the added bio-yogurt of *Bacillus subtilis* as probiotic culture.

*TP – Total peptides derived from bio-yogurt; CV - Coefficient of variation; Different lowercase letters on the same line differ from each other in relation to the lactose-probiotic system; Different capital letters on the same line results differ statistically in relation to the weight of the peptides.

Hydroxyl (%)					
System	Lactose	Probiotic	TP*	10-3 kDa	3 kDa
01	Absence	Absence	100.00 aA	100.00 aA	100.00 aA
02	Presence	Absence	100.00 aA	94.85 aAB	87.19 bB
03	Absence	Presence	100.00 aA	100.00 aA	100.00 aA
04	Presence	Presence	100.00 aA	95.00 aA	100.00 aA
CV (%) = 4.39					
Superoxide (%)					
01	Absence	Absence	95.88 aA	99.19 aA	99.10 aA
02	Presence	Absence	62.44 bB	74.52 bA	58.60 bB
03	Absence	Presence	60.81 bB	93.69 aA	56.96 bB
04	Presence	Presence	96.40 aA	97.09 aA	97.49 aA
CV (%) = 3.59					

Table 4: Antioxidant activity of scavenging of the hydroxyl and superoxide radicals of the total bioactive peptides of the bio-yogurt and its fractions.

TP - Total peptides derived from bio-yogurt; CV - Coefficient of variation; Different lowercase letters on the same line differ from each other in relation to the lactose-probiotic system; Different capital letters on the same line results differ statistically in relation to the weight of the peptides.

It is known that antioxidants play an important role in increasing shelf life of foods, inhibiting lipid oxidation, and protecting macromolecules, such as proteins and vitamins [30]. The results of the present study indicate that the peptides present in *Bacillus subtilis* containing bio-yogurt present a high antioxidant potential and can increase shelf life. In addition, at the gastrointestinal level the peptides with lower molecular weight may cross the intestinal wall without any hydrolytic action [31].

Conclusion

Peptides obtained from bio-yogurt produced using lactose and lactose - free milk in the presence or absence of probiotic (*Bacillus subtilis*) showed ABTS, DPPH, superoxide, hydroxyl and chelation of iron and copper. Peptides of different sizes and systems differed statistically ($p<0.05$) between some assays. In addition, good industrial yield was observed. Thus, *Bacillus subtilis* shows a promising probiotic in the production of bio-yogurt with antioxidant potential and increased shelf life. In addition, the peptides of the lactose-free and probiotic-free systems can still be used as nutraceutical ingredients in functional foods.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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