



Effect of *in vitro* gastrointestinal digestion on the antioxidant potential of yogurt added with probiotic culture containing *Bacillus subtilis*

Efeito da digestão gastrointestinal *in vitro* no potencial antioxidante de iogurte adicionado de cultura probiótica contendo *Bacillus subtilis*

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ABSTRACT: The addition of probiotic culture confers potential bio functional to food because of the ability to promote health, and the beneficial effects in the body. The objective was to produce with lactose and lactose-free yogurt with *Bacillus subtilis* addition and to evaluate the antioxidant potential of water-soluble peptides during the storage period. For yogurt production, a simple factorial arrangement was used to evaluate two types of milk, adding probiotic culture containing *B. subtilis* UFPEDA 86. The fractions were evaluated (pre-digestion and post-digestion) concerning the antioxidant elimination potential of the radical acid 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2'-diphenyl 1-picrylhydrazyl (DPPH), chelation of copper and iron. In the evaluation of the results, the fractions obtained from the different combinations of milk, probiotic culture and different days presented antioxidant potential and chelating capacity, activities showed a significant increase after the digestion process. In addition, the results demonstrated that the *B. subtilis* could support the production of bio-yogurt with antioxidant potential.

KEYWORDS: functional foods, healthy eating, lactose-free milk

RESUMO: A adição de cultura probiótica confere potencial biofuncional aos alimentos, devido à capacidade de promover a saúde e aos efeitos benéficos no organismo. O objetivo foi produzir iogurte com lactose e sem lactose com adição de *Bacillus subtilis* e avaliar o potencial antioxidante de peptídeos solúveis em água durante o período de armazenamento. Para a produção de iogurte, foi utilizado um arranjo fatorial simples para avaliar dois tipos de leite com adição da cultura probiótica contendo *B. subtilis* UFPEDA 86. As frações foram avaliadas (pré-digestão e pós-digestão) quanto ao potencial antioxidante de eliminação do radical 2,2'-azino-bis (ácido 3- etilbenzotiazolina -6-ácido sulfônico) (ABTS), do 2,2'-difênil-1-picril-hidrazil (DPPH) e quelação de cobre e ferro. Na avaliação dos resultados, as frações obtidas das diferentes combinações de leite, cultura probiótica e dias de armazenamento apresentaram potencial antioxidante e capacidade quelante; as atividades apresentaram aumento significativo após o processo de digestão. Além disso, os resultados demonstraram que o *B. subtilis* pode ser empregado na produção de bio-iogurte com potencial antioxidante.

PALAVRAS-CHAVE: Alimento funcional, alimentação saudável, leite sem lactose.

INTRODUÇÃO

The addition of ingredients in foods with the function of potentializing their nutritional actions has been growing over the last few years (MOUMITA *et al.*, 2018). This addition confers potential bio functional to food because of the ability to promote health, and the biochemical and physiological effects in the body (STAKA; BODNIEKS; PUĶĪTIS, 2015; ANGELOV *et al.*, 2018).

Among the various types of fermented functional foods, Probiotics are defined as 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (HILL *et al.*, 2014). There are several fermented drinks, beverages and medicines that were widely consumed (ANGMO *et al.*, 2016; SEKAR; VINOTHKANNA, 2019) of which the bio-yogurt is an efficient vehicle to receive the addition of living microorganism.

Probiotics are beneficial microorganisms that have innumerable biological applications, emphasizing utilization to strengthen and maintain the intestinal barrier and keep it healthy (STAKA; BODNIEKS; PUĶĪTIS, 2015); prevention of cardiovascular diseases (MOUMITA *et al.*, 2018); blood pressure control (MIREMADI; SHERKAT; STOJANOVSKA, 2016); proteolytic activity (MADHU; AMRUTHA; PRAPULLA, 2012); antioxidant activity (SHI *et al.*, 2019), among others.

The antioxidant activity occurs through the elimination of free radical compounds, which originates from the metabolism of living organisms, which are the cause of premature aging, which can cause cancer, diabetes and other diseases to humans (CHAVAN *et al.*, 2011). The addition of the strain of *Bacillus subtilis*, has as main objective the expansion of the functions of yogurt, as well as the antioxidant function and consequently its improvement and quality (VINOTHKANNA; SEKAR, 2019).

B. subtilis is a GRAS (generally regarded as safe) gram-positive and nonpathogenic bacillus that can be found on soil. Literature reposts several studies about the safe use of this bacterial species (ELSHAGHABEE *et al.*, 2017). Among these applications are the use in food-grade enzyme production (GHANI *et al.*, 2013; OUATTARA *et al.*, 2017) and additional nutraceuticals such as riboflavins, cobalamin, inositol and carotenoids (MOHAMMED *et al.*, 2014; TANAKA; TAKANAKA, 2014; TAKANO, 2016). Besides

that, *B. subtilis* is widely employed in Africa for the production of fermented beverages and seasonings (NATH; CHOWDHURY; DORA, 2015).

In this context, this work aimed to evaluate the scavenging capacity of oxidative radicals' compounds and the chelation of metallic ions of the fractions of probiotic yogurt produced through the interactions between the presence and absence of lactose and addition of *Bacillus subtilis* in its composition, in different days, before and after simulation of *in vitro* digestion.

MATERIALS AND METHODS

YOGURT PREPARATION

Ultra-high temperature (UHT) milk of bovine origin with lactose and lactose-free, pasteurized were subjected to an additional heat treatment (91 ± 1 °C / 10 min). Then, the milk was cooled to 45 °C and the lyophilized starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* was inoculated at the concentration of 0.6 g.L⁻¹ and the probiotic culture constituted of *Bacillus subtilis* UFPEDA 86, provided by the Antibiotics Department of Universidade Federal de Pernambuco (UFPE), at concentration of 0.25 g.L⁻¹ for probiotic culture. The fermentation was carried out at 45 ± 1 °C for a period of 12 hours (Table 1) and the final point of the yogurt fermentation was based on the clot firmness check. Subsequently, the product was cooled to 4 ± 1 °C and the clot was broken by manual shaking with glass a rod.

Table 1. Experimental design of the factorial arrangement of treatments (2 x 2)

System	Definition
System 1 (S1)	With lactose and fermented with addition of <i>Bacillus subtilis</i>
System 2 (S2)	With lactose and fermented without addition of <i>Bacillus subtilis</i>
System 3 (S3)	Lactose-free and fermented with addition of <i>Bacillus subtilis</i>
System 4 (S4)	Lactose-free and fermented without addition of <i>Bacillus subtilis</i>

ABTS^{•+} RADICAL SCAVENGING ASSAY

The antioxidant activity assay involving the scavenging of the cation ABTS^{•+} radical, generated from the oxidation of 2,2'-azinobis-3-ethylbenzothiazoline-6-acid sulfonic (ABTS) 7 mM with persulphate potassium 2.45 mM, pre-incubated in the dark for 12 hours prior to use. The ABTS^{•+} solution was adjusted from 0.700±0.02 to 734 nm of absorbance in a spectrophotometer, by dilution in buffer phosphate 5 mM, performed according to the methodology described by Hernández-Ledesma *et al.* (2014). An aliquot of 50 µL of the sample was mixed with 950 µL diluted solution of ABTS^{•+}; this reaction mixture was incubated for 10 minutes in the dark at room temperature (23 °C). The absorbance of the reaction was measured at 734 nm and the ABTS^{•+} radical scavenging assay was calculated according to the equation 1:

$$\text{ABTS radical scavenging (\%)} = \left[\left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \right] * 100 \quad \text{Eq.1}$$

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

DPPH[•] RADICAL SCAVENGING ASSAY

The antioxidant activity assay involving the scavenging of DPPH[•] (2,2-diphenyl-1,4-phenylenediamine) radical was determined according to the methodology described by Yen and Chen, (1995). The reaction mixture consisted of 200 µL of the sample and 200 µL of 0.16 mM ethanolic solution 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 DPPH[•] radical scavenging assay of the was calculated using the equation 2:

$$\text{DPPH radical scavenging (\%)} = \left[1 - \left(\frac{A_{\text{sample}} - A_{\text{White}}}{A_{\text{control}}} \right) \right] * 100 \quad \text{Eq.2}$$

Where A_{sample} is the absorbance of the samples, $A_{white\ sample}$ is the absorbance of the sample without DPPH[•], and $A_{control}$ is the absorbance of the control sampled DPPH solution.

CHELATING ACTIVITY OF Fe²⁺ AND Cu²⁺ OF THE FRACTIONS OF THE PEPTIDES

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 and pH 4.9) and 12.5 µL of Fe²⁺ (2 mM). After 30 minutes of incubation, 50 µL of ferrozine solution (5 mM) was added after a further 30 min and then read on a spectrophotometer at 562 nm (SÁNCHEZ-VIOQUE *et al.* 2012).

The copper chelating activity was performed with modifications employing 0.5 mL of sodium acetate buffer (pH 6.0, 50 mM) and mixed with 12.5 µL CuSO₄ 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 min of incubation. The absorbance of the reaction mixture was evaluated at 632 nm in a spectrophotometer (SAIGA *et al.*, 2003).

For both activities, the negative control was performed using water in substitution of the sample; the positive control was performed with 0.045% EDTA solution, the percentage of inhibition was determined according to the equation 3:

$$CA(\%) = \left[\frac{(A_{control} - A_{sample})}{A_{control}} \right] * 100 \quad \text{Eq. 3}$$

DIGESTION *IN VITRO*

The digestion experiment was performed simulating the physiological conditions of the human organism in the stomach and intestine, adapted from a method already described by Gião *et al.* (2012). The volume of each infusion was divided equally by 3

bottles of Schott glass, one of the vials wrapped in aluminum foil and placed in the cold (sample digested, 4 °C) and the other two underwent a gastric digestion (duplicates), with the correctness of its pH to 2.0 using 1 M HCl and 0.05 mL of pepsin solution (25 mg.mL⁻¹ in 0.1 M HCl) per mL of sample. The samples were then wrapped and incubated in a 37 °C bath for two hours under shaking at 100 rpm digestion, away from the light.

After the incubation period, the gastric digestion test tubes were submitted to intestinal digestion, with their pH adjusted to 6.0 using 1M NaHCO₃ and 0.25 mL of the mixed solution of pancreatin and bile salts (2 g.L⁻¹ of pancreatin and 12 g.L⁻¹ bovine bile in 1 M NaHCO₃). The samples wrapped in foil were incubated at 37 °C bath for one hour, under shaking at 45 rpm. After determination of the volume of the digested samples and of which the larger volume flask, ultra-pure water was added to all other flasks (undigested samples), so that all presented with the same final volume. All samples underwent a heat shock enzymatic inactivation process by submersion in water at 100 °C for 4 minutes, followed by cooling by ice storage for 10 minutes. At the end of this period, the samples were again filtered with quantitative paper filter and stored in sterile microtubes.

STATISTICAL ANALYSIS

The parameters were analyzed in triplicate and the data expressed as mean and standard deviation. The experimental design was a factorial (2 x 2), totaling 4 systems, according to Frame 1. Significant differences between the 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 Tukey multiple comparison test in SISVAR version 5.6 (FERREIRA, 2008).

RESULTS AND DISCUSSION

The bio-yogurt showed the potential of elimination of the ABTS +[•] and DPPH+[•] radicals in all systems throughout the storage period (Table 2). The S3 system (lactose-free and fermented with *Bacillus subtilis* addition) showed the highest scavenging capacity of this radical, remaining between 68.77 and 91.30%, corroborate to the results found by Lee *et al.* (2018) with the elaboration of yogurt with the addition of *Lactobacillus plantarum*. The lowest percentage was observed in the experiments that presented lactose in its composition when compared to lactose-free yogurt.

In relation to the elimination of the DPPH+[•] radical, the systems with a time of 0 day of storage presented the highest percentage of scavenging of the radical with decreased activity throughout the days, the biochemical properties of some protein hydrolysates and their respective bioactive peptides have strong antioxidant activities in the water-soluble radicals elimination system (ABTS+[•]), but not in the soluble lipid system (DPPH+[•]), as observed for the protein hydrolysates of the present study (BARBA *et al.*, 2013), differing from the results found by Madhu, Amrutha and Prapulla (2012). where over time the results showed an increase in the activity. This can be explained because there is a higher concentration of antioxidant peptides, at time 0 compared to the following days.

Tests carried out in the absence of lactose in the presence or absence of *Bacillus subtilis* showed a higher elimination potential of the radical, S2 (63.52%) and S1 (58.58%), respectively (Table 2). The occurrence of good activity in lactose-free yogurt is of great relevance since cases of lactose intolerance have been increasing over the years (XIONG *et al.*, 2017; MANUYAKORN; TANPOWONG, 2019; OAK; JHA, 2018).

Table 2. ABTS[•] and DPPH[•] radical scavenging and potential chelating activity of Fe²⁺ and Cu²⁺ bio-yogurt with lactose and lactose-free in the presence of probiotic culture containing *Bacillus subtilis* without *in vitro* digestion process.

ABTS[•]				
	0 day	7 days	14 days	21 days
S1	89.28 ^{Aa}	61.53 ^{Cb}	65.61 ^{Bb}	56.07 ^{ABb}
S2	81.61 ^{Aa}	32.88 ^{ABb}	48.49 ^{Bb}	42.33 ^{Bb}
S3	91.30 ^{Aa}	76.15 ^{Aab}	88.43 ^{Aab}	68.77 ^{Ab}
S4	82.55 ^{Aa}	50.75 ^{BCb}	48.40 ^{Bb}	40.83 ^{Bb}
DPPH				
	0 days	7days	14 days	21 days
S1	58.58 ^{Abc}	16.78 ^{Ba}	0.00 ^{Cc}	2.77 ^{Cb}
S2	39.18 ^{Ac}	0.78 ^{Cb}	10.63 ^{Bbc}	13.53 ^{Ba}
S3	63.52 ^{Aa}	0.15 ^{Cb}	1.49 ^{Cbc}	13.19 ^{Ba}
S4	49.98 ^{Ab}	0.00 ^{Cb}	11.25 ^{Ba}	2.11 ^{Bb}
IRON CHELATING				
	0 days	7 days	14 days	21 days
S1	2.49 ^{Bb}	87.61 ^{Aa}	87.57 ^{Aa}	86.70 ^{Aa}
S2	1.74 ^{Bb}	85.66 ^{Aa}	85.41 ^{Aa}	85.49 ^{Aa}
S3	5.98 ^{Ba}	86.41 ^{Aa}	86.58 ^{Aa}	86.78 ^{Aa}
S4	4.70 ^{Bab}	85.12 ^{Aa}	85.16 ^{Aa}	84.54 ^{Aa}
COPPER CHELATING				
	0 days	7 days	14 days	21 days
S1	88.91 ^{Aa}	90.23 ^{Aa}	89.97 ^{Aa}	90.73 ^{Aa}
S2	89.77 ^{Aa}	91.81 ^{Aa}	90.60 ^{Aa}	90.14 ^{Aa}
S3	87.56 ^{Aa}	91.41 ^{Aa}	86.60 ^{Aa}	90.46 ^{Aa}
S4	77.76 ^{Bb}	91.48 ^{Aa}	91.23 ^{Aa}	92.74 ^{Aa}

S1- Lactose-free system and without *Bacillus subtilis*; S2- With lactose system and without *Bacillus subtilis*; S3- Lactose-free system and with *Bacillus subtilis*; S4- Lactose-free system and with *Bacillus subtilis*. Different capital letters on the same line indicate statistical difference between the times ($p < 0.05$); Different lowercase letters in the same column indicate statistical difference between trials ($p < 0.05$).

It is known that high levels of iron and copper ions in the body lead to oxidative stress and consequently cause cellular damage and disease (PRESA *et al.*, 2018). Regarding the iron chelating activity, a lower elimination potential was observed in the first storage period with an increase during the storage time. There was no significant difference between the times and the systems after 7, 14 and 21 days of storage with activity greater than 80%.

The chelation of copper remained above 85% in almost all the tests, with no significant difference between the times and the systems. Presa *et al.* (2018) indicate that the treatment with synthetic chelating agents cause great side effects and their

applications in the industrial sectors are under specific legislation that the limit their use, therefore, it is necessary to prospect for new natural antioxidant agents, such as bioactive peptides. After bio-yogurt submission to the simulation of the *in vitro* digestion process, it was observed that the antioxidant and chelating potential of metal ions was maintained in most of the trials (Table 3).

Table 3. ABTS⁺•, DPPH• radical scavenging and potential chelating activity of Fe²⁺ and Cu²⁺ bio-yogurt of lactose-free and lactose-free yogurt with probiotic culture containing *Bacillus subtilis* after *in vitro* digestion.

ABTS ⁺ •				
	0 days	7 days	14 days	21 days
S1	98.49 ^{Ab}	99.44 ^{Aa}	98.17 ^{Aab}	99.39 ^{Aab}
S2	84.52 ^{Bb}	98.59 ^{Aa}	98.40 ^{Aa}	98.97 ^{Aa}
S3	98.40 ^{Ab}	98.59 ^{Aa}	98.73 ^{Ab}	99.44 ^{Aa}
S4	99.01 ^{Aab}	98.16 ^{Aa}	99.06 ^{Aa}	99.34 ^{Aa}
DPPH•				
	0 days	7 days	14 days	21 days
S1	15.07 ^{Abc}	14.57 ^{Aa}	14.83 ^{Aa}	15.61 ^{Abc}
S2	23.06 ^{Aa}	17.29 ^{ABa}	15.09 ^{Aa}	12.46 ^{Acb}
S3	12.92 ^{Abc}	13.99 ^{Aa}	8.39 ^{Ba}	5.96 ^{Bc}
S4	22.41 ^{Abc}	20.86 ^{Aa}	15.39 ^{Aa}	21.72 ^{Aa}
IRON CHELATING				
	0 days	7 days	14 days	21 days
S1	84.25 ^{Aa}	78.60 ^{Bb}	79.22 ^{Bb}	78.05 ^{Ba}
S2	84.25 ^{Aa}	83.58 ^{Aa}	82.92 ^{ABab}	79.26 ^{Ba}
S3	86.49 ^{Aa}	86.66 ^{Aa}	85.04 ^{Ab}	77.72 ^{Ba}
S4	73.03 ^{Cb}	84.41 ^{ABa}	86.12 ^{Ab}	80.59 ^{Ba}
COPPER CHELATING				
	0 days	7 days	14 days	21 days
S1	25.06 ^{Bb}	38.53 ^{Ac}	17.90 ^{Bb}	13.45 ^{Cb}
S2	0.00 ^{Ac}	0.00 ^{Aa}	0.00 ^{Ac}	0.00 ^{Ac}
S3	18.06 ^{Bb}	22.80 ^{ABb}	29.44 ^{Aa}	29.81 ^{Aa}
S4	35.16 ^{Aa}	20.02 ^{Bb}	34.03 ^{Aa}	23.24 ^{Ba}

S1- Lactose free system and without *Bacillus subtilis*; S2 - System with lactose and without *Bacillus subtilis*; S3 - Lactose free system and with *Bacillus subtilis*; S4 - System with lactose and with *Bacillus subtilis*. Different capital letters on the same line indicate statistical difference between the times ($p < 0.05$). Different lowercase letters in the same column indicate statistical difference between the trials ($p < 0.05$).

The scavenging potential of the ABTS⁺• radical was between 84.52% and 99.44% among the systems maintained in different storage periods. An increase in the post-

digestion radical scavenging potential was observed. This indicates that digestive enzymes act by releasing peptides with ABTS⁺• scavenging activity, as found in the work of McCarthy *et al.* (2015), adding grains, which are used in the preparation of beer which caused an increase in antioxidant activity after the digestion process, a fact that did not occur with "pure" yogurt, addition of ingredients to yogurts is necessary because of the increase in their biological activity (JIN *et al.*, 2016)

In relation to the DPPH⁺• radical scavenging assay, it was observed that after the *in vitro* digestion the tests at zero storage time showed a reduction of about 50% in the radical scavenging activity. On the other days of storage increase in the capacity to free radical scavenging when compared to the same tests before the simulation.

The release of post-digestive bioactive peptides may be responsible for the increase results in these articles. According to Jin *et al.* (2016), the peptides released by the post-digestion yogurt can increase biological activities, among them the scavenging of ABTS⁺• and DPPH⁺• radicals.

The iron chelation capacity in the periods of 7, 14 and 21 days of storage remained statistically the same and did not present post-digestion difference. There was a decrease in the ability to chelate copper after the digestion simulation, highlighting the S1 (between 13.45 and 38.53%) and S3 (between 18.06 and 29.81%) trials that presented the greatest activities between trials.

CONCLUSION

The prospecting of bioactive peptides from yogurt produced using milk with lactose and lactose-free in the presence or not of probiotic (*Bacillus subtilis*) demonstrates efficacy against the ability to scavenge radicals' oxidants (ABTS⁺• and DPPH⁺•) and potential chelating of copper and iron metal ions. The lactose-free systems show peptides with higher potential to eliminate the radicals ABTS⁺•, DPPH⁺•, and chelating iron and copper. *Bacillus subtilis* is a promising probiotic in the production of yogurt with antioxidant potential and shelf life. The antioxidant potential of the yogurt produced *in vitro* using *B. subtilis* was reported. However, for future use in the industry, studies about toxins production must be evaluated.

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