Journal of Food Composition and Analysis 69 (2018) 162–170

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original research article

Lactobacillus fermentation of jussara pulp leads to the enzymatic conversion of anthocyanins increasing antioxidant activity

Anna Rafaela Cavalcante Braga[a], Leonardo Mendes de Souza Mesquita[a,b], Paula Larangeira Garcia Martins[a], Sascha Habu[a], Veridiana Vera de Rosso[a,*]

[a] Department of Bioscience, Federal University of São Paulo – UNIFESP, Silva Jardim Street, 136, Santos, São Paulo State, ZIP Code: 11015-020, Brazil
[b] Biosciences Institute, São Paulo State University – UNESP, Coastal Campus of São Vicente, Pça Infante Dom Henrique S/N, 11330 – 900 São Vicente, SP, Brazil

ARTICLE INFO

Chemical compounds studied in this article:
Cyanidin 3-glucoside (PubChem CID: 92131208)
Cyanidin 3-rutinoside (PubChem CID: 44256716)
Protocatechuic acid (PubChem CID: 72)
3,4-dihidroxyphenylpropionic acid (PubChem CID: 161525)
m-hydroxyphenylacetic acid (PubChem CID: 12122)
p-coumaric acid (PubChem CID: 637542)
Caffeic acid (PubChem CID: 689043)
Ferulic acid (PubChem CID: 445858)

Keywords:
β-glucosidase
Cyanidin 3-glucoside
Cyanidin 3-rutinoside
Experimental design
Fermentation
Jussara
Food analysis

ABSTRACT

Bacteria possessing an enzymatic system able to metabolize anthocyanins may play a major role in the production of compounds with different bioavailability and biological activity. In this study, *Lactobacillus* and *Bifidobacterium* strains were screened for the enzymatic activities of α-galactosidase, β-galactosidase and β-glucosidase. These strains were also screened for their ability to convert the anthocyanins from jussara. The nine evaluated strains produced at least two of the three enzymes; they were also capable of changing the main anthocyanin's chromatographic profile. The values of cyanidin 3-glucoside and cyanidin 3-rutinoside, following fermentation, ranged from 0 to 3217.7 and 4323.3 to 17190.9 μg/100 mL of medium fermented, respectively. The *Lactobacillus deubruekii* strain was able to change the anthocyanins from jussara pulp more extensively. Therefore, a culture medium containing jussara pulp and glucose was optimized using the experimental design as a statistical tool. Medium maximization occurred with a mixture composing 20% jussara pulp and 10% glucose in which the microorganism was able to reach the highest enzymatic production as well as produce the most extensive conversion of the main anthocyanins. Protocatechuic acid was the main enzymatic bioconversion product identified following fermentation. In addition, there was an observed increase in antioxidant activity following the jussara pulp fermentation process.

1. Introduction

Jussara (*Euterpe edulis* Mart.) is a palm tree found throughout the Atlantic Forest in Brazil. The fruit of jussara has a rounded shape and is dark purple, which is due to the anthocyanin content. Jussara fruit is similar to the açai berry in both its sensorial and nutritional properties. Studies in our group showed that jussara supplementation during pregnancy and lactation modulates gene and protein expression of inflammation biomarkers and improves colonic expression of ZO-1 in offspring (Morais et al., 2016, 2014). Additionally, jussara supplementation improves glucose tolerance in mice (Oyama et al., 2016). The high concentration of phenolic compounds, especially anthocyanins, appears to be responsible for these biological effects.

Anthocyanins have antioxidant properties, acting as free radical scavengers in *in vitro* models, and are also frequently linked to the prevention of cardiovascular diseases (Borges et al., 2013). Moreover, anthocyanins are interesting due to their technological applications as natural colorants or antioxidants, as well as to their ability to be used as food additives in several products (Ávila et al., 2009).

Despite the beneficial properties of anthocyanins, their effectiveness at preventing or treating a range of diseases depends on their bioavailability, and it is this group of pigments that is less well absorbed from the gastrointestinal tract than other flavonoids (Faria et al., 2014). Additionally, the biological activity of anthocyanins can also be affected by glycosylation, which renders the molecule more water-soluble but less reactive towards free radicals and metals, thereby diminishing their antioxidant activity (Correia et al., 2012). Therefore, bacteria possessing an enzymatic system able to metabolize anthocyanins may

* Corresponding author.
E-mail addresses: annarafaela@gmail.com (A.R.C. Braga), mesquitalms@gmail.com (L.M.d.S. Mesquita), paula.sfc@gmail.com (P.L.G. Martins), sashabu@yahoo.com.br (S. Habu), veriderosso@yahoo.com, veridiana.rosso@unifesp.br (V.V. de Rosso).

https://doi.org/10.1016/j.jfca.2017.12.030
Received 21 November 2016; Received in revised form 12 October 2017; Accepted 20 December 2017
Available online 24 December 2017

play a major role in the production of compounds with different bioavailabilities and biological activities (Ávila et al., 2009), which is because hydrolysis of anthocyanin glycosides is proposed as the first step for subsequent bacterial degradation and the formation of a set of new metabolites (Faria et al., 2014).

Microorganisms belonging to the genera *Bifidobacteria* and *Lactobacillus* are predominant members of the intestinal microbiota, and some strains have been characterized as probiotics. It has been shown that species of *Bifidobacteria* and *Lactobacillus* possess β-glucosidase activity and participate in the hydrolysis of plant β-glycosides (Ávila et al., 2009; Matsuda et al., 1994), but little is known about their potential for biotransforming anthocyanins. The findings of both *in vitro* and *in vivo* studies suggest a potential anti-inflammatory effect of these compounds, which seem to inhibit the activation of the signaling pathway, which is mediated by the transcription factor NF-κB. This effect is associated with the modulation of a beneficial gut microbiota, particularly an increase in *Bifidobacterium strains* (Morais et al., 2016).

The role of the interaction between the microbiota and anthocyanins, and the bioconversion of these compounds into phenolic acids could explain much of the lack of information in regards to this aspect in the literature. However, each subject has his/her own microbiota, it clearly interferes with the bioavailability of anthocyanins due to interindividual differences (Braga et al., 2018). One way to standardize the beneficial effects of anthocyanins and their metabolites is to offer the population products fermented in controlled conditions, granting in this way that, even without the most health intestinal microbiota, the benefits will be provided by these regularly consumed products.

Considering the great potential of jussara pulp, due to its of anthocyanins content and the ability of microorganisms to bioconvert these compounds, the objectives of this study were to screen *Bifidobacteria* and *Lactobacillus* strains for β-glucosidase activity and to investigate their enzymatic potential for converting the main anthocyanins from jussara pulp into other metabolites. This way, we would acquire the knowledge regarding the possibility of pre-fermented products being used as a model for improvement of bioacccessibility of anthocyanins and their metabolites. In addition, the antioxidant activity was evaluated before and after the jussara pulp fermentation.

2. Material and methods

2.1. Sample

The jussara pulp was obtained directly from producers linked to the Jussara Project from Ubatuba City – São Paulo – Brazil. The frozen jussara pulp was transported in coolers and stored in a freezer until the analysis.

2.2. Bacterial strains and growth conditions

Bifidobacterium dentium ATCC 27534, *Bifidobacterium lactis* CCT 7503, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus delbrueckii* ATCC 4796, *Lactobacillus fermentum* ATCC 15442, *Lactobacillus paracasei* ATCC 335, *Lactobacillus plantarum* ATCC 10012, *Lactobacillus rhamnosus* ATCC 7469, and *Lactobacillusbrevis* ATCC 367 were obtained from the Oswaldo Cruz Foundation (FIOCRUZ) culture collection. The cultures were maintained at −40 °C in MRS broth supplemented with glycerol (20% v/v) and subcultured in MRS broth before their use in the experiments.

2.3. Chemicals

HPLC-grade methanol and formic acid were obtained from Merck (Darmstadt, Germany). The other reagents were all analytical grade obtained from Labsynth (Diadema, Brazil). The samples and solvents were filtered through Millipore (Billerica, MA, USA) membranes (0.22 and 0.45 μm) before HPLC analyses. The standards for cyanidin 3-glucoside (98.9%), cyanidin 3-rutinoside (98.0%), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (99.0%) were purchased from Sigma-Aldrich (Darmstadt, Germany).

The purity of these standards was determined by HPLC-DAD. The standards were used as received. The other chemicals *p*-nitrophenyl-β-D-galactopyranoside (pNPG), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), *n*-nitrophenyl-β-D-glucopyranoside, a,a-azodiisobutyramidinedihydrochloride (AAPH), fluorescein, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (Darmstadt, Germany).

2.4. Jussara pulp fermentation

The cultures for inoculum were grown on MRS broth in flasks with 50 mL of culture medium. The sterilization of the medium was performed at 121 °C for 15 min. The cultures were incubated in an orbital shaking incubator for 24 h at 100 rpm and 28 °C. The optical density of each culture was standardized at OD = 0.500 of absorbance at 600 nm by spectrophotometry, and the inoculum was standardized at 2% (v/v). A control assay was conducted, applying the same conditions of the experiments, in order to guarantee that all the composition changes were as a consequence of the microbial activity during the fermentation. In order to select the microorganism that could provide the greatest bioconversion of anthocyanins from jussara pulp, the submerged cultivations were performed in conical flasks. The cultivation medium for screening was composed of 20% jussara pulp added to 10% glucose, due to the low concentration of fermentable sugars in the jussara pulp (1.5%) (Rogez, 2000), and the pH was adjusted to 5.6 (the natural pH of the pulp). A 200 mL volume of culture medium was distributed to 500-ml flasks and heat-treated by flowing steam at 100 °C for 13 min to maintain the bioactive compounds. The operational conditions were 28 °C and 100 rpm for 48 h, and the cultivations were conducted in triplicate for each microorganism. All analyses were performed in triplicate. At appropriate intervals, culture samples of the α-galactosidase, β-galactosidase, and β-glucosidase activities were collected for analyses. Following 48 h of cultivation, the major anthocyanins were also measured.

2.5. Experimental design to maximize a culture medium containing jussara pulp

For the experimental design, submerged cultivations were conducted on a medium containing jussara pulp and glucose. The component concentrations changed according to the experimental design. A Central Composite Rotatable Design (CCRD; 2^2 plus axial and central points) with three replicates at the central point, giving a total of 11 trials, was used, with β-glucosidase activity and bioconversion (%) of the main anthocyanins of jussara pulp (cyanidin 3-glucoside and cyanidin 3-rutinoside) being the responses. Table 1 shows the values for the real and coded levels used in the CCRD. Statistica 12.0 software was used to analyze the results. The enzymatic activities were measured every 24 h of cultivation for up to 48 h. For each trial, the response from the experimental designs was the maximum enzymatic activity and bioconversion (%) of the main anthocyanins of jussara pulp following 48 h of cultivation. An estimate of the main effect was

Table 1
Real and coded values used in the Central Composite Design Rotational (CCDR).

Coded variable level	Jussara pulp (%)	Glucose (%)
−1.41	10.0	0
−1	12.9	2.9
0	20.0	10.0
+1	27.1	17.1
+1.41	30.0	20.0

obtained by evaluating the difference in process performance caused by a change from the low (−1) to the high (+1) levels of the corresponding variable.

2.6. Anthocyanin determination

The anthocyanins were extracted from jussara pulp before and after the fermentation process using 100 mL of 0.5% HCl in methanol overnight at 5 °C in darkness. The homogenate was filtered and concentrated in a rotary evaporator (T < 38 °C) in order to provide the crude extracts (De Rosso and Mercadante, 2007a; Da Silva et al., 2014). The crude extract was diluted with water containing 5% formic acid/methanol (85:15, v/v) immediately before analysis by HPLC-DAD. The levels of anthocyanins were determined using a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps (model LC-20AD), a DAD detector (Shimadzu, model SPD-M20A), a degasser unit (DGU-20A5), and a Rheodyne injection valve with a 20-μL loop (De Rosso and Mercadante, 2007a; Da Silva et al., 2014) and were therefore quantified by HPLC-DAD using seven-point analytical curves of cyanidin 3-glucoside (5–125 μg/mL) and cyanidin 3-rutinoside (10–200 μg/mL). The concentration was expressed in μg of cyanidin 3-glucoside/mL and/or μg of cyanidin 3-rutinoside/mL. The anthocyanins were identified based on the combined information provided by elution order in the C_{18} reversed phase column and cochromatography with the standards, as well as UV − visible and mass spectra in comparison with the literature data.

The anthocyanins were quantified using the percentage of the area obtained during the HPLC-DAD analysis (De Rosso and Mercadante, 2007a, 2007b; Da Silva et al., 2014). All of the analyses were performed in triplicate. To evaluate the degree of anthocyanin enzymatic conversion, the rate was calculated using Eq. (1):

$$\%Bioconversion = \frac{Initial\ Anthocyanins}{Final\ Anthocyanin} x\ 100 \quad (1)$$

2.7. Determination of enzymatic activities

Enzymatic activities of three enzymes (α-galactosidase, β-galactosidase and β-glucosidase) were determined during the fermentation time. α-galactosidase activity was measured spectrophotometrically following the recommendations of the Food Chemicals Codex (2003), using *p*-nitrophenyl-β-D-galactopyranoside (pNPG), and one unit of enzyme activity (U) was defined as the quantity of enzyme that liberates 1 μmol of *p*-nitrophenol per min under the assay conditions. The β-galactosidase activity was measured spectrophotometrically using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Inchaurrondo et al., 1994). One unit of enzyme activity (U) was defined as the quantity of enzyme that liberates 1 μmol of *o*-nitrophenol per min under the assay conditions.

Finally, the β-glucosidase activity (intra- and extracellular) was measured by releasing *p*-nitrophenol from *n*-nitrophenyl-β-D-glucopyranoside (Matsuda et al., 1994), and one unit of enzyme activity (U) was defined as the quantity of enzyme that liberates 1 μmol of *p*-nitrophenol per min under the assay conditions.

2.8. Kinetics of jussara pulp fermentation

The best culture medium obtained was prepared in order to evaluate the kinetics of the jussara pulp fermentation. The medium containing jussara pulp was aseptically inoculated with the greatest bacteria bioconversion of anthocyanins and incubated at 28 °C/100 rpm for 48 h. Samples were collected at the following times (hours): 0, 1, 2, 4, 6, 8, 12, 24 and 48, and the determinations for the bioconversion of anthocyanins (De Rosso and Mercadante, 2007b, 2007a) and β-glucosidase activity were obtained.

2.9. Mass spectrometry

Samples were collected during fermentation process at the following times (hours): 0, 1, 2, 4, 6, 8, 12, 24 and 48, the aim being to monitor the metabolites formed. These samples were extracted using 100 mL of 0.5% HCl in methanol overnight at 5 °C in darkness, the homogenate was filtered and concentrated in a rotary evaporator (T < 38 °C) to provide the crude extracts (De Rosso and Mercadante, 2007a; Da Silva et al., 2014). Furthermore, the extracts were injected by directed infusion using mass spectrometry in an attempt to identify the main products generated from the *Lactobacillus deubruekii* action.

A mass analysis was performed using electrospray ionization (ESI–MS/MS) LTQXL Thermo Ion Trap (San Jose, CA). Extracts were injected by directed infusion at 10 μL/min flow rate, using a MS parameters: dry gas, N_2 at 10 psi, with a spray voltage of 4.52 kV, a capillary voltage of 17 V and at a source temperature of 280 °C. Positive mode ionization was used to investigate the anthocyanins, and the negative and positive mode was used for the phenolic acids. The FIA-ESI-IT-MS/MS collision energy was between 20 and 30% (2 eV). The MS/MS spectrum was obtained between 50 and 1000 *m/z*. The data acquisition software used was ThermoXcalibur.

2.10. Antioxidant activity assays in jussara pulp in natura and fermented

An extract was prepared from jussara pulp (before and after fermentation, at zero and 48 h) with 100 mL of 80% cold acetone by agitation with a magnetichomogenizer (Tecnal, Piracicaba, Brazil) for 15 min; the slurry was filtered, and the solids were washed twice with an additional 100 mL of 80% acetone and then concentrated in a rotary evaporator (T < 40 °C).

The antioxidant activity against the $ABTS^{\cdot+}$ radical was measured by adding an acetone/water extract (30 μL) to a diluted solution of $ABTS^{\cdot+}$ (7 mM); the solution was homogenized and after 6 min, the absorbance was read at 734 nm and compared with a known Trolox standard curve (Re et al., 1999). The results were expressed as μmol of Trolox equivalent/100 g of raw sample. The antioxidant activity against the peroxyl radical ($ROO^{\cdot}$) was measured by the ORAC assay, which is based on monitoring the effect of the hydrophilic extract orstandard (Trolox) on the fluorescence decay that results from $ROO^{\cdot}$ inducedoxidation of fluorescein (Rodrigues et al., 2012). The $ROO^{\cdot}$ was generated by thermodecomposition of AAPH at 37 °C. The assay was performed in a 96-wells microplate containing fluorescein (61 nM) prepared in phosphate buffer 75 mM, pH 7.4), AAPH solution (19 mM) in phosphatebuffer, hydrophilic extract in three different dilutions (100, 500 and 1000 times) in phosphate buffer, or Trolox (50 μM) in phosphate buffer. The results were expressed as μmol of Trolox equivalent/100 g of raw sample.

3. Results and discussion

3.1. Bacterial screening for jussara pulp fermentation

A previous experiment was conducted, using the same conditions of the experiments realized, to evaluate the possibility there being a chemical biotransformation of the anthocyanins due to pH or other conditions, and the results showed that the same amount of both anthocyanins were found after 48 h considering the medium with jussara pulp and glucose (without the inoculation of any microorganisms). Therefore, we can affirm that the changes in the anthocyanins' profile were results of the microbial metabolism during the fermentation process.

When shaken flask cultures were used to investigate the enzymatic bioconversion of major anthocyanins from jussara pulp by the nine bacterial strains, the enzymatic activities shown in Table 2 were observed. This study shows that each evaluated strain presented the activity of at least two of the enzymes tested. The main enzyme evaluated

Table 2
Maximum enzymatic activities and main anthocyanin levels of the nine microorganisms during cultivation.

Microorganism	β-galactosidase MA (U/mL)	α-galactosidase MA (mU/mL)	β-glucosidase MA (mU/mL)	C3G (μg/100 mL)*	C3R (μg/100 mL)*
B. dentium	1.283^{c}	55.4^{a}	38.3^{a}	1774.2^{b}	17190.9^{a}
B. lactis	1.967^{b}	22.7^{b}	6.90^{d}	383.0^{cd}	8534.3^{d}
L. acidophilus	0.020^{f}	0^{d}	0.01^{e}	419.8^{c}	11302.7^{c}
L. brevis	1.070^{d}	49.3^{a}	34.9^{b}	3217.7^{a}	14349.9^{b}
L. deubruekii	0.987^{de}	55.3^{a}	23.7^{c}	0.0^{d}	4323.3^{e}
L. fermentum	0.883^{e}	19.6^{b}	10.1^{d}	361.9^{c}	10989.9^{c}
L. paracasei	2.273^{a}	20.7^{b}	9.93^{d}	421.0^{c}	10487.8^{c}
L. plantarum	0.987^{de}	0^{d}	1.13^{e}	602.1^{c}	13565.6^{b}
L. rhamnosus	1.144^{cd}	10.7^{c}	40.5^{a}	545.4^{c}	9945.4^{cd}

MA: Maximum Activity; C3G: Cyanidin 3-glucoside; C3R: Cyanidin 3-rutinoside. * The concentration was expressed in μg/100 mL of medium after the fermentation process. Different letters in the same column represent significantly different values ($p < 0.05$).

in this case was β-glucosidase because it participates in the hydrolysis of plant β-glycosides, which could improve the bioacccessibility of anthocyanins. Since the majority of dietary anthocyanins are not absorbed at the upper gastrointestinal level (Faria et al., 2014), the enzymes' action over anthocyanins, especially the β-glucosidase, causes the biotransformation into their metabolites, which can be better absorbed.

The maximum activity for β-galactosidase was obtained by *L. paracasei*, *B. lactis* and *B. dentium*, reaching values of up to 2.3 U/mL. The greatest producers of α-galactosidase were *B. dentium*, *L. brevis* and *L.deubruekii,* and these strains presented 55.4, 49.3 and 55.3 mU/mL, respectively. The strains *B. dentium*, *L. brevis* and *L. rhamnosus* showed the highest productions of β-glucosidase, reaching values up to 40.5 mU/mL. The values of cyanidin3-glucoside and cyanidin3-rutinoside following fermentation ranged from 0 to 3217.7 and 4323.3 to 17190.9 μg/100 mL of medium fermented, respectively (Table 2). The nine evaluated strains were able to convert the major anthocyanins from jussara pulp. The most significant decrease in cyanidin3-glucoside and cyanidin3-rutinoside levels after a 48-h fermentation period was shown when *L. deubruekii* was evaluated. *L. brevis* and *B. dentium*, were the microorganisms that changed the least amounts of anthocyanins following the fermentation period. Following 48 h of fermentation, the remaining viable microbiota (all strains) were determined through a plate count at 30 °C of between 1.5×10^{6}cfu mL^{-1} and 2.4×10^{8} cfu mL^{-1}.

Several fermentation processes utilize combinations of microbial strains in an attempt to determine a synergistic effect between them, thus enhancing the enzymatic machinery, which is especially true for biotechnological purposes. In this sense, the co-cultivation of the *Lactobacillus* and *Bifidobacterium* strains, which showed the most promising results (*B. lactis*, *L. deubruekii* and *L. rhamnosus*), were assessed in an attempt to maximize the effect on the anthocyanins from jussara pulp. The results of the maximum enzymatic activities obtained from the co-culture assays and the results of the main anthocyanin levels (cyanidin 3-glucoside and cyanidin 3-rutinoside) for the co-cultures following the fermentation process are presented in Table 3. Co-cultures using *B. lactis*and *L. rhamnosus* showed the highest enzymatic activity for α-galactosidase (37.7 mU/mL) and β-galactosidase (1.57 U/mL). The highest value obtained for β-glucosidase activity was obtained using *B. lactis* and *L. deubruekii* strains for jussara pulp fermentation.

The main anthocyanin values decreased more extensively considering the co-culture of *B. lactis* and *L. deubruekii*. However, despite the great results obtained using co-culture fermentation, the strain *L. deubruekii* alone reached the same, or even better, results. Therefore, *L. deubruekii* was considered the best strain to maximize production of β-glucosidase and anthocyanin bioconversion using the experimental design.

The enzymatic activities measured were not linearly responsible for the breakdown of anthocyanins, and it is difficult to correlate these results, since the highest enzymatic activities did not always lead to a greater bioconversion of the anthocyanins. However, it is mandatory to remember that there is a large enzymatic complex responsible for the microbial metabolism, therefore, it is extremely possible that the action of other enzymes, which were not measured in this study, acted simultaneously. Due to the metabolic richness of the evaluated bacteria, it would be difficult to measure the enzymatic activity of all the enzymes present in the medium, so this lack of correlation between the amount of enzymes evaluated and the changes in anthocyanin profiles is quite plausible.

3.2. Experimental design to maximize a culture medium containing jussara pulp

Table 4 shows the results obtained for β-glucosidase enzymatic activity, cyanidin 3-glucoside bioconversion (%) and cyanidin 3-rutinoside bioconversion (%) in the CCRD with the two variables studied: jussara pulp (%) (X1) and glucose (%) (X2). The terms that were not statistically significant were incorporated into the lack of fit for calculation of the R^2 and F ratio.

The enzyme production varied from 12.9 to 44.0 mU/mL, and the best values for activity occurred in trials 4 and 6. The conditions in trial 4 were jussara pulp (%) at level +1 (27.1) and glucose (%) at level +1 (17.1). In this trial, the activity reached 42.7 mU/mL after 24 h of fermentation. In trial 6, the conditions were jussara pulp (%) at level +1.41 (30.0) and glucose (%) at level 0 (10.0). In this trial, the maximum enzymatic activity was 44.0 mU/mL after 24 h of cultivation. The main effects and interactions were estimated for β-glucosidase activity and resulted in Eq. (2).

$$\beta - \text{glucosidase enzymatic activity (mU/mL)} = 32.9 + 10.5(X1) - 2.0(X1)^2 + 1.5(X2)^2 \quad (2)$$

ANOVA was used to evaluate the adequacy of the fitted model. Thus, the maximum values of β-glucosidase activity were used in this

Table 3
Maximum enzymatic (MA) activities of the co-cultures and values of the main anthocyanin levels for the co-cultures after the fermentation process.

Microorganism	β-galactosidase MA (U/mL)	α-galactosidase MA (mU/mL)	β-glucosidase MA (mU/mL)	C3G (μg/100 mL)*	C3R (μg/100 mL)*
B. lactis/L. deubruekii	1.32^{b}	11.4^{a}	15.4^{b}	0.0^{b}	4487.8^{b}
B. lactis/L. rhamnosus	1.57^{a}	37.7^{b}	23.4^{a}	498.9^{a}	9201.0^{a}

* The concentration was expressed in μg/100 mL of medium after the fermentation process.

Table 4
Real, coded values and experimental values for enzymatic activity and bioconversion of major anthocyanins in the CCRD.

Run	Jussara pulp (%)	Glucose (%)	β-glucosidase activity (mU/mL)	C3G bioconversion (%)	C3R bioconversion (%)
1	−1 (12.9)	−1 (2.9)	18.9	86.5	43.5
2	+1 (27.1)	−1 (2.9)	39.3	90.8	31.7
3	−1 (12.9)	+1 (17.1)	23.6	88.4	48.3
4	+1 (27.1)	+1 (17.1)	42.7	91.9	40.0
5	−1.41 (10)	0 (10)	12.9	82.0	12.7
6	+1.41 (30)	0 (10)	44.0	84.5	34.0
7	0 (20)	−1.41 (0)	31.4	90.4	52.0
8	0 (20)	+1.41 (20)	33.9	92.1	51.8
9	0 (20)	0 (10)	32.4	100.0	53.1
10	0 (20)	0 (10)	32.7	100.0	50.0
11	0 (20)	0 (10)	33.2	100.0	51.7

C3G: Cyanidin 3-glucoside; C3R: Cyanidin 3-rutinoside.

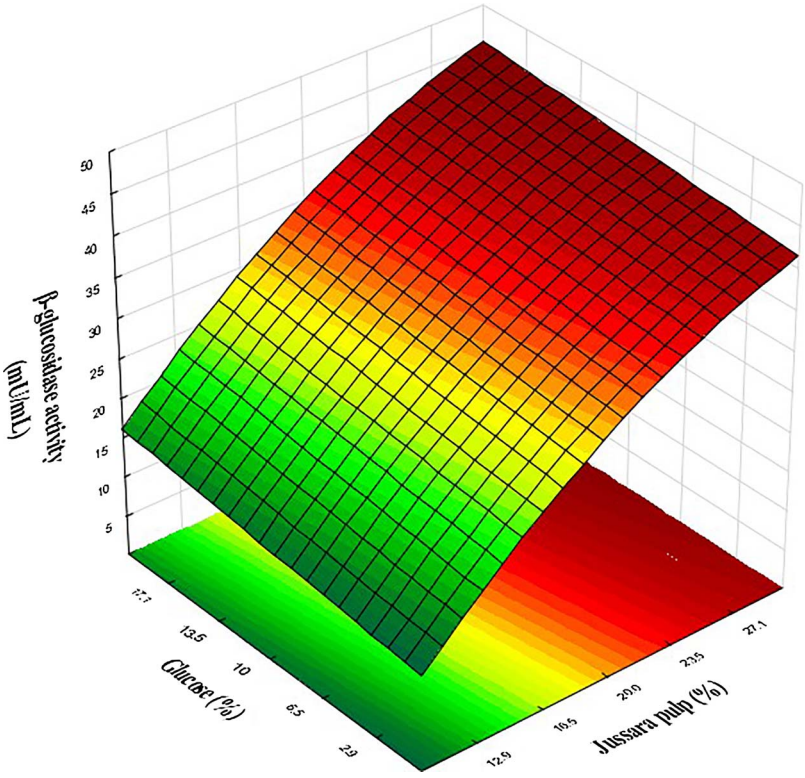

Fig. 1. Response surface for β-glucosidase activity as a function of the concentrations of jussara pulp and glucose.

analysis. The R^2 value provided a measurement of how much the variability in the observed response values in Fig. 1 could be explained by the experimental factors and their interactions. A good model (values above 0.9 are considered very good) explains most of the variation in the response. The closer the R^2 value is to 1.00, the stronger the model and the better the response predictions are (Alves et al., 2010; Braga et al., 2012). In this study, an R^2 value of 0.98 was obtained. The F value was approximately three times the F value tabulated for β-glucosidase activity, exceeding the 95% confidence level.

The central points provided additional degrees of freedom for error estimation, which increased the power when testing the significance of effects (Alves et al., 2010). In our work, the pure error was very low, indicating good reproducibility of the experimental design. Thus, the coded model was considered predictive, and it can be used to generate the response surface for the ANOVA effects on β-glucosidase activity according to the model equation (Eq. (2)). An overview of Eq. (2) indicates that the activity is a first-order function of the jussara pulp (%) and glucose (%) and a second-order function of the jussara pulp (%). Eq. (2) shows the equation model fitted according to the regression analysis. Experimental design it is a great statistical tool that uses regression analysis and is widely used in bioprocessing, especially in culture medium optimization (Braga et al., 2012; Machado et al., 2015).

The model for β-glucosidase activity was used to construct the response surface observed in Fig. 1, the objective of which was to understand the interactions between the medium components and the optimum concentration of each component required for maximization of β-glucosidase production. Fig. 1 shows that when the jussara pulp (%) value is high, the glucose (%) has almost no effect on enzyme production, and the highest enzyme activities (mU/mL) are reached using the maximum jussara pulp (%) values. This result shows the importance of the jussara pulp concentration on enzyme activity.

Despite the enzymatic activity being very important, as it enables the partial breakdown of the anthocyanins, which may increase their bioavailability and bioacccessibility, the bioconversion of the main anthocyanins from jussara pulp should be carefully evaluated in conjunction with enzyme activity. Therefore, the experimental design results of the percentage of bioconversion of the main anthocyanins were obtained, and these data are presented in Table 4.

The bioconversion percentage ranged from 82 to 100% and from 12.7 to 53.1% for cyanidin 3-glucoside and cyanidin 3-rutinoside, respectively.

The highest bioconversion percentage for both major anthocyanins was observed in trials 9, 10 and 11. The conditions in those trials were jussara pulp (%) at level 0 (20.0) and glucose (%) at level 0 (10.0), corresponding to the central points. Coincidentally, these concentrations were used in the screening assays, indicating that this step had already been performed in the maximized conditions for the bioconversion of anthocyanins present in the jussara pulp. The main effects and interactions were estimated for the bioconversion percentage of cyanidin 3-glucoside and cyanidin 3-rutinoside, resulting in Eqs. (3) and (4).

$$\text{cyanidin 3 - glucoside bioconversion (\%)} = 99.9 + 1.4\,(X1) - 7.9\,(X1)^2 - 3.8\,(X2)^2 \quad (3)$$

$$\text{cyanidin 3 - rutinoside bioconversion (\%)} = 52.5 - 13.7\,(X1)^2 \quad (4)$$

Considering the data provided from the ANOVA table calculated for both bioconversion of anthocyanin models, the R^2 values found were 0.94 and 0.81 for cyanidin 3-glucoside and cyanidin 3-rutinoside, respectively. The F values were significant at approximately five and three times the F values tabulated for cyanidin 3-glucoside and cyanidin 3-rutinoside, respectively, exceeding the 95% confidence level.

The models for cyanidin 3-glucoside and cyanidin 3-rutinoside were used to construct the response surface observed in Fig. 2a and b. Fig. 2a shows that when the jussara pulp (%) and the glucose (%) are at the central point's value, maximum bioconversion (%) is achieved. Additionally, Fig. 2b shows that when the jussara pulp (%) value is at the central point, the glucose (%) has almost no effect on the bioconversion of cyanidin 3-rutinoside.

To increase β-glucosidase production and the bioconversion of major anthocyanins from jussara pulp, the authors chose to work with the conditions indicated by the surfaces using the following culture medium: 20% jussara pulp and 10% glucose. Fig. 3 shows the average results for the kinetic fermentation, performed in triplicate, using the fermentation conditions described above.

Fig. 3a shows that for α-galactosidase and β-glucosidase, enzymatic activities increased up to 24 h of cultivation, reaching 63.0 and 42.7 mU/mL, respectively. The activity for the β-galactosidase enzyme was not detected during the cultivation time. Fig. 3b presents the bioconversion percentages ofcyanidin 3-glucoside and cyanidin 3-rutinoside, which reached bioconversions of 100 and 58%, respectively. The highest bioconversion percentage for both major anthocyanins occurred at 48 h, and these values are very similar to those found in the best trials from the CCDR (assays 9, 10 and 11). Therefore, it was possible to validate the model obtained from the experimental design.

3.3. Formed metabolites after bioconversion of anthocyanins from jussara

The bioconversion of anthocyanins from jussara pulp by *L. deubruekii* metabolism was evident. The products obtained from the fermentation process were determined using mass spectrometry for identification and are presented in Table 5. The anthocyanins identified in the jussara pulp were previously reported (Da Silva et al., 2014). In the fermentation process, each anthocyanin is converted into phenolic compounds of a lower molecular weight. The free aglycone were not detected as intermediate metabolites at any time of fermentation, therefore it is extremely important to quantify and characterize the compounds formed in order to recognize which substance is responsible for the beneficial human health effect that are related to anthocyanins and their metabolite consumption. This fact is probably because the formation of phenolic acids occurs simultaneously with the fragmentation of sugars moieties, since they are already being formed in the early fermentation times (from 2 h). The probable pathway of anthocyanins bioconversion in phenolic acids is through chalcones, as proposed by Faria et al. (2014).

In addition, Xie et al. (2016), proposes various pathways of phenolic acids in an *in vivo* system, through flavonoids such as catechin, quercetin and anthocyanins. Furthermore, phenolic acids can be converted to each other with small modifications. The caffeic acid, (*m/z* 178), can be transformed into 3,4-dihydroxyphenylpropionic acid (*m/z* 181) (Supplementary material); as it can be converted into protocatechuic acid (*m/z* 153) (Fig. 4). All these mechanisms are quite reasonable, as they are detected from the same fermentation time (Table 5). Phenolic compounds generated from cyanidin 3-glucoside, cyanidin 3-rutinoside and cyanidin 3-pentose-hexose are protocatechuic acid (*m/z* 153 – Fig. 4), 3,4-dihidroxyphenylpropionic acid (*m/z* 181 – Supplementary material) and caffeic acid (*m/z* 178 – Supplementary material), according to Xie et al. (2016), who identified the same colonic metabolites following consumption of aronia berry. Two perlagonidin and two phenolic compounds were detected by FIA-ESI-IT-M/MS^2. Perlagonidin 3-glucoside and perlagonidin 3-rutinoside were bioconverted into *p*-coumaric acid (*m/z* 162 – Supplementary material) and m-hydroxyphenylacetic acid (*m/z* 162 – Supplementary material). Peonidin 3-rutinoside is bioconverted in ferulic or isoferulic acid (*m/z* 194 – Supplementary material).

The bioconversion of anthocyanins in phenolic acids was also observed in *in vivo* studies. Xie et al. (2016) observed spontaneous degradation of anthocyanins by the action of bacteria that make up the microbial flora. Early *in vitro* studies on the metabolism of anthocyanins

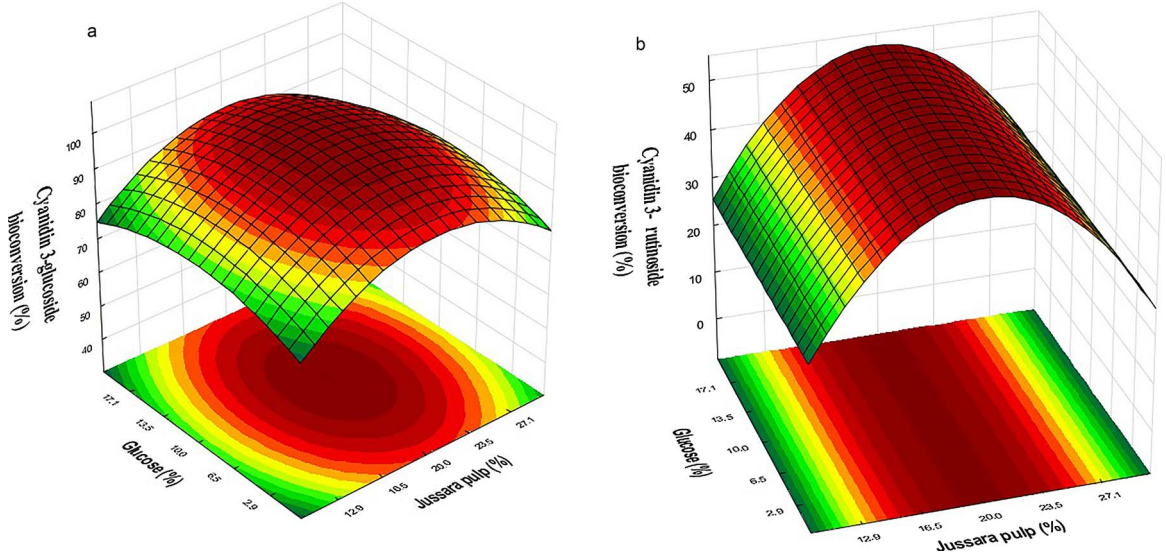

Fig. 2. Response surface for (a) cyanidin 3-glucoside bioconversion (%) and (b) cyanidin 3-rutinoside bioconversion (%) as a function of the concentrations of jussara pulp and glucose.

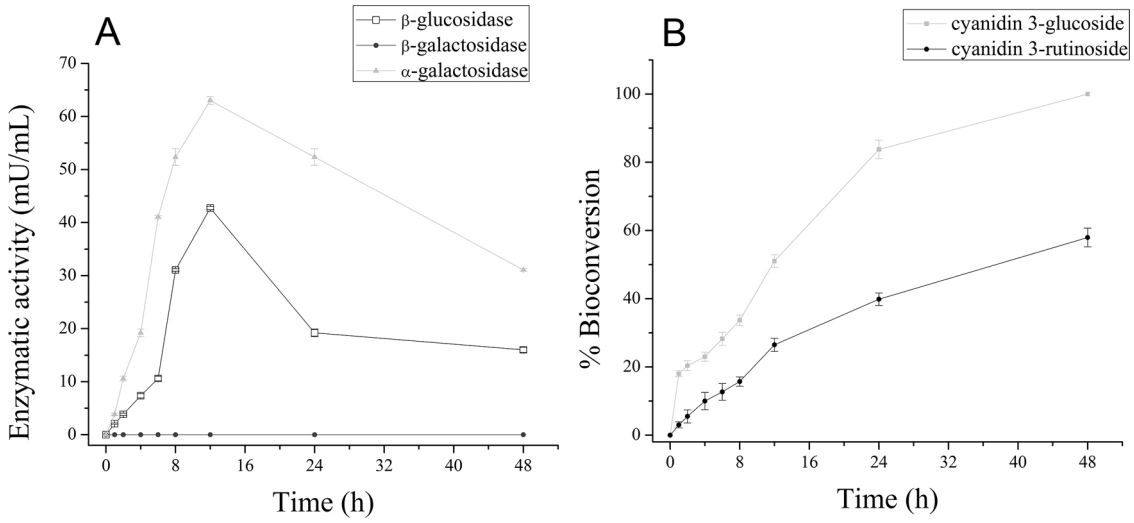

Fig. 3. Best conditions cultivation using *Lactobacillus deubruekii* during cultivation time: (a) profiles for enzymatic activities and (b) profiles for anthocyanins bioconversion.

Table 5
Mass spectrometry characteristics of anthocyanins and phenolic acids from jussara fermentation medium.

Anthocyanins	$[M+H]^+$	MS/MS fragments	Kinetics of fermentation times (h)								
			0	1	2	4	6	8	12	24	48
cyanidin 3-glucoside	449	287	1	1	1	1	1	1	0	0	0
cyanidin 3-rutinoside	595	449 287	1	1	1	1	1	1	1	1	1
cyanidin 3,5-hexose pentose	581	449 287	1	1	1	1	0	0	0	0	0
pelargonidin 3-glucoside	433	271	1	1	1	1	1	0	0	0	0
pelargonidin 3-rutinoside	579	433 271	1	1	1	1	1	0	0	0	0
peonidin 3-rutinoside	609	463 301	1	1	1	1	1	0	0	0	0
Phenolic acids	**$[M+H]^-$**	**MS/MS fragments**	**Kinetics of fermentation times (h)**								
			0	1	2	4	6	8	12	24	48
protocatechuic acid	153	97 109 125	0	0	1	1	1	1	1	1	1
3,4-dihidroxyphenylpropionic acid	181	109 137 153 166 181	0	0	1	1	1	1	1	1	1
m-hydroxyphenylacetic acid	152	95 110 122	0	0	1	1	1	1	1	1	1
p-coumaric acid	162	104 118	0	0	1	1	1	1	1	1	1
caffeic acid	178	134 150	0	0	1	1	1	1	1	1	1
ferulic acid and isoferulic acid	194	134 150 194	0	0	0	0	0	1	1	1	1

1 = detected; 0 = not detected.

by gut microflora have concluded that bacterial metabolism involved the cleavage of glycosidic linkages and the breakdown of anthocyanidin heterocycle (Aura et al., 2004), and, based on the data obtained, the same seems to have occurred in our study. In addition, in accordance with the literature, protocatechuic acid is the major human metabolite of cyanidin-glucosides (Faria et al., 2014; Vitaglione et al., 2007).

3.4. Antioxidant activity assays in natura and fermented jussara pulp

The antioxidant activity in jussara before and after the fermentation process is reported by the antioxidant activity assays for ABTS and peroxyl radicals (ORAC) in order to determinate if the change of anthocyanins composition affected this parameter. Considering the ABTS assay, fermented jussara resulted in notably significant increases in antioxidant activity compared to jussara pulp *in natura*, from 142.1 to 212.0 μmol TE/100 g of sample (an increase of 49.2%; $p < 0.00000$). This effect was also confirmed by the ORAC assay, enhancing the antioxidant activity from 46.3 to 88.3 μmol TE/100 g of sample (an increase of 90.7%; $p < 0.000134$), therefore, the fermented jussara showed an increase of antioxidant activity compared with the *in natura* pulp in both antioxidant assays. Saxena et al. (2012) reports that the antioxidant activity depends on the number of free hydroxyl groups in the molecular structure. Thus, the bioconversion of anthocyanins in phenolic acids is an important process in the increased activity.

Changes in the anthocyanins profile following the fermentation process, in addition to the increase antioxidant activity, could be considered strong evidence in regards to the micro-organisms' role in helping enhance the beneficial properties of anthocyanins. Additionally, the fermentation process shows an extremely promising potential in developing food products using fruits rich in anthocyanins, improving their properties and opening a wide range of applications in the food industry. The next step following these results is to evaluate the bioavailability of jussara pulp before and after the fermentation process. Additionally, in order to improve scientific knowledge to complement the results found in our study, a food model could be produced and used as a tool to verify the increase of bioacccessibility and bioavailability of pre-fermented anthocyanins.

4. Conclusions

According to the findings shown in this study, it is evident that enzymes produced by bacteria can transform anthocyanins from jussara pulp. To the best of our knowledge, this is the first study reporting the enzymatic potential of single strains of *Lactobacillus* and *Bifidobacterium*

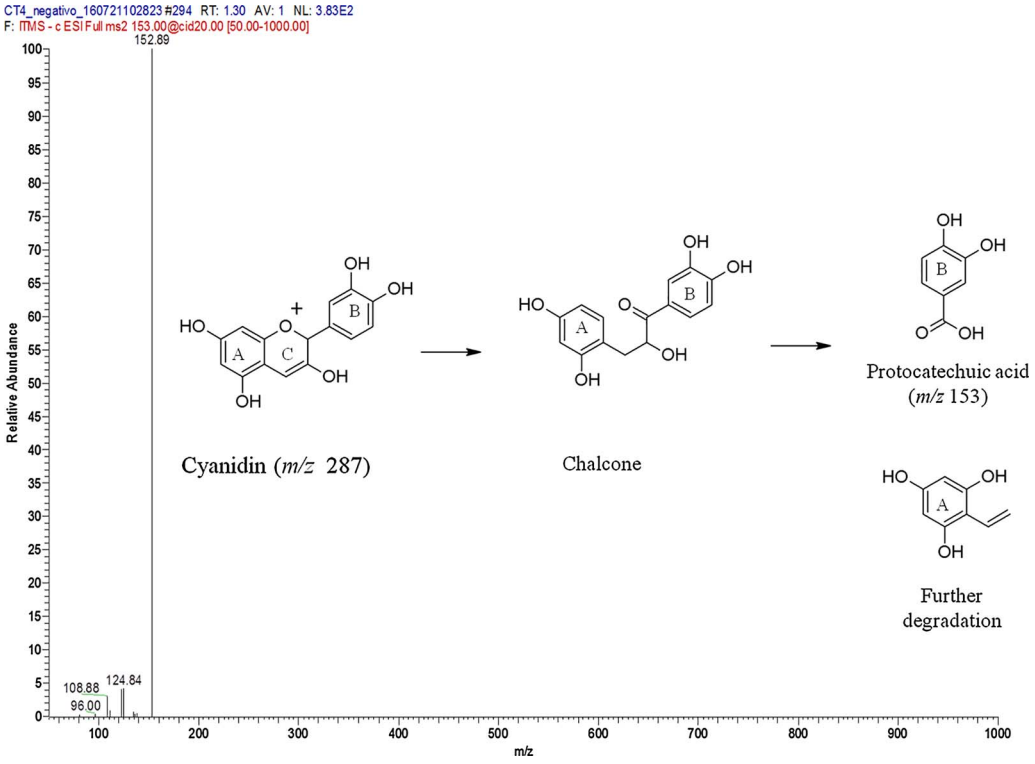

Fig. 4. Bioconversion of cyanidin into protocatechuic acid via fermentation process.

for bioconversion of anthocyanins from jussara pulp. In addition, a culture medium containing jussara pulp and glucose was elaborated and optimized using an experimental design. The metabolites generated from the bioconversion of cyanidin3-glucoside and cyanidin3-rutinoside must be further evaluated to determine the ideal degree of anthocyanin conversion to improve their bioacccessibility. Finally, an increase in antioxidant activity was observed following the fermentation process, which shows a great application potential of these processes for developing new products using fruits containing anthocyanins.

Acknowledgment

The authors acknowledge the financial support of grant No. 2014/09301-6, São Paulo Research Foundation (FAPESP).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jfca.2017.12.030.

References

Ávila, M., Hidalgo, M., Sánchez-Moreno, C., Pelaez, C., Requena, T., de Pascual-Teresa, S., 2009. Bioconversion of anthocyanin glycosides by Bifidobacteria and Lactobacillus. Food Res. Int. 42, 1453–1461. http://dx.doi.org/10.1016/j.foodres.2009.07.026.

Alves, F.G., Maugeri, F., Burkert, J.F.M., Kalil, S.J., 2010. Maximization of β-galactosidase production: a simultaneous investigation of agitation and aeration effects. Appl. Biochem. Biotechnol. 160, 1528–1539.

Aura, A.M., Martin-Lopez, P., O'Leary, K.A., Williamson, G., Oksman-Caldentey, K.M., Poutanen, K., Santos-Buelga, C., 2004. In vitro metabolism of anthocyaninsby human gut microflora. Eur. J. Nutr. 44, 133–142. http://dx.doi.org/10.1007/s00394-004-0502-2.

Borges, G.D.S.C., Gonzaga, L.V., Jardini, F.A., Mancini Filho, J., Heller, M., Micke, G., Costa, A.C.O., Fett, R., 2013. Protective effect of Euterpe edulis M. on Vero cell culture and antioxidant evaluation based on phenolic composition using HPLC – ESI–MS/MS. Food Res. Int. 51, 363–369. http://dx.doi.org/10.1016/j.foodres.2012.12.035.

Braga, A.R.C., Gomes, P.A., Kalil, S.J., 2012. Formulation of culture medium with agroindustrial waste for β-galactosidase production from *Kluyveromyces marxianus* ATCC 16045. Food Bioprocess Technol. 5, 1653–1663.

Braga, A.R.C., Murador, D.C., Mesquita, L.M. de S., De Rosso, V.V., 2018. Bioavailability of anthocyanins: Gaps in knowledge, challenges and future research. J. Food Compos. Anal., in press.

Correia, R.T., Borges, K.C., Medeiros, M.F., Genovese, M.I., 2012. Bioactive compounds and phenolic-linked functionality of powdered tropical fruit residues. Food Sci. Technol. Int. 18, 539–547.

Da Silva, N.A., Rodrigues, E., Mercadante, A.Z., De Rosso, V.V., 2014. Phenolic compounds and carotenoids from four fruits native from the Brazilian Atlantic Forest. J. Agric. Food Chem. 62, 5072–5084. http://dx.doi.org/10.1021/jf501211p.

De Rosso, V.V., Mercadante, A.Z., 2007a. The high ascorbic acid content is the main cause of the low stability of anthocyanin extracts from acerola. Food Chem. 103, 935–943. http://dx.doi.org/10.1016/j.foodchem.2006.09.047.

De Rosso, V.V., Mercadante, A.Z., 2007b. HPLC–PDA–MS/MS of anthocyanins and carotenoids from Dovyalis and Tamarillo fruits. J. Agric. Food Chem. 55, 9135–9141. http://dx.doi.org/10.1021/jf071316u.

Faria, A., Fernandes, I., Norberto, S., Mateus, N., Calhau, C., 2014. Interplay between anthocyanins and gut microbiota. J. Agric. Food Chem. 62, 6898–6902. http://dx.doi.org/10.1021/jf501808a.

Inchaurrondo, V.A., Yantorno, O.M., Voget, C.E., 1994. Yeast growth and b-galactosidase production during aerobic batch cultures in lactose-limited synthetic medium. Process Biochem. 29, 47–54.

Machado, J.R., Behling, M.B., Braga, A.R.C., Kalil, S.J., 2015. β-Galactosidase production using glycerol and byproducts: whey and residual glycerin. Biocatal. Biotransform. 33, 208–215. http://dx.doi.org/10.3109/10242422.2015.1100363.

Matsuda, S., Norimoto, F., Matsumoto, Y., Ohba, R., Teramoto, Y., Ohta, N., Ueda, S., 1994. Solubilization of a novel isoflavone glycoside-hydrolyzing β-glucosidase from Lactobacillus casei subsp. rhamnosus. J. Ferment. Bioeng. 77, 439–441. http://dx.doi.org/10.1016/0922-338X(94)90021-3.

Morais, C.A., Oyama, L.M., de Oliveira, J.L., Garcia, M.C., De Rosso, V.V., Amigo, L.S.M., Nascimento, C.M.O., do Pisani, L.P., 2014. Jussara (Euterpe edulis Mart.) supplementation during pregnancy and lactation modulates the gene and protein expression of inflammation biomarkers induced by trans-fatty acids in the colon of offspring. Mediators Inflamm. 1–11.

Morais, C.A., De Rosso, V.V., Estadella, D., Pisani, L.P., 2016. Anthocyanins as inflammatory modulators and the role of the gut microbiota. J. Nutr. Biochem. 33, 1–7. http://dx.doi.org/10.1016/j.jnutbio.2015.11.008.

Oyama, L.M., Silva, F.P., Carnier, J., de Miranda, D.A., Santamarina, A.B., Ribeiro, E.B., Oller do Nascimento, C.M., De Rosso, V.V., 2016. Juçara pulp supplementation improves glucose tolerance in mice. Diabetol. Metab. Syndr. 8, 8. http://dx.doi.org/10.1186/s13098-015-0122-4.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26, 1231–1237. http://dx.doi.org/10.1016/S0891-5849(98)00315-3.

Rodrigues, E., Mariutti, L.R.B., Faria, A.F., Mercadante, A.Z., 2012. Microcapsules containing antioxidant molecules as scavengers of reactive oxygen and nitrogen species. Food Chem. 134, 704–711. http://dx.doi.org/10.1016/j.foodchem.2012.02.163.

Rogez, H., 2000. Açaí preparo, composição e melhoramento da conservação. EDUFPA, Belém, PA.

Saxena, M., Saxena, J., Pradhan, A., 2012. Flavonoids and phenolic acids as antioxidant in plants and human health. Int. J. Sci. Rev. Res. 16, 130–134.
Vitaglione, P., Donnarumma, G., Napolitano, A., Galvano, F., Gallo, A., Scalfi, L., Fogliano, V., 2007. Protocatechuic acid is the major human metabolite of cyanidin-glucosides. J. Nutr. 137, 2043–2048.
Xie, L., Lee, S.G., Vance, T.M., Wang, Y., Kim, B., Lee, J.-Y., Chun, O.K., Bolling, B.W., 2016. Bioavailability of anthocyanins and colonic polyphenol metabolites following consumption of aronia berry extract. Food Chem. 211, 860–868. http://dx.doi.org/10.1016/j.foodchem.2016.05.122.