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Antioxidant and prebiotic effects of a beverage composed by tropical fruits and yacon in alloxan-induced diabetic rats

Ana Paula DIONISIO[1]*, Luciano Bruno de CARVALHO-SILVA[2], Nara Menezes VIEIRA[3], Nedio Jair WURLITZER[1], Ana Carolina da Silva PEREIRA[4], Maria de Fatima BORGES[1], Deborah dos Santos GARRUTI[1], Idila dos Santos ARAÚJO[1]

Abstract

The aim of this study was to evaluate the antioxidant and prebiotic effect of a beverage composed by tropical fruits and yacon in alloxan-induced diabetic rats. To obtain the beverage, a Response Surface Methodology was used, with concentration of yacon extract (x1) and the sweetener (x2) added to a mixture of tropical fruits as independent variables, and the sensorial acceptance as response. The optimized beverage showed higher values for antioxidant capacity, measured by ABTS, FRAP and DPPH assays. Thus, the fructooligosaccharides content (2.32 ± 0.65 g FOS/100 g), total phenolic (126.83 ± 9.48 mg gallic acid equivalent/100 g) and ascorbic acid (171.64 ± 7.31 mg/100 g) shows that the beverage contains high levels of these bioactive compounds. In addition, the beverage was tested in vivo, using male Wistar rats, divided into five groups (control, non-treated diabetic, and diabetic treated with 100, 200 or 400 mg of lyophilized beverage/kg/day). The results showed a promotion of the growth of lactobacilli in cecal material and an increase in catalase activity in the liver, in a dose-dependent manner, showing the prebiotic and antioxidant effects of the beverage in the diabetic model.

Keywords: diabetes; oxidative estress; antioxidants; tropical fruits; prebiotics.

Practical Application: In this work we developed a beverage with prebiotic and antioxidant properties.

1 Introduction

The International Scientific Association for Probiotics and Prebiotics (ISAPP) defines a prebiotic as "[...] a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017, p. 493). Besides they are naturally present in plant sources like chicory, onion and garlic (Farias et al., 2019; Mohanty et al., 2018; Quigley, 2019), the yacon (*Smallanthus sonchifolius*) – a Andean tuberous root – is one of the most abundant source of fructooligosaccharides (FOS) which exhibit prebiotic properties (Al-Sheraji et al., 2013).

Recently, studies have shown that the yacon possess different biological effects, among immunomodulation (Delgado et al., 2012), antimicrobial (Ojansivu et al., 2011) and antioxidant (Sousa et al., 2015b; Campos et al., 2012). In addition, studies have reported that the ingestion of yacon reduces glycemia and increases the concentration of insulin in the plasma (Park et al., 2009; Aybar et al., 2001; Satoh et al., 2013) and reduces the glycemia of diabetic rats (Dionisio et al., 2015). Moreover, in clinical assays, the yacon have also to be associated with subjective improvements in satiety and reductions in post prandial glucose and insulin concentrations (Adriano et al., 2019; Silva et al., 2017; Kellow et al., 2014 – see also the references listed).

The American Diabetes Association (2010, p. S62) defines diabetes mellitus as "[...] a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both". High blood glucose concentrations promote auto-oxidation of glucose to form free radicals. The generation of free radicals beyond the scavenging abilities of endogenous antioxidant defenses results in macro-and microvascular dysfunction and polyneuropathy (Bajaj & Khan, 2012).

Cells have evolved highly complex enzymatic (*e.g.*, catalase) and non-enzymatic antioxidant systems (*e.g.*, ascorbic acid and polyphenols), which work synergistically, and in combination with each other, to protect the body against free radical-induced damage. There are several lines of evidence to suggest that antioxidant defenses may be lower in diabetes (Karunakaran & Park, 2013). In this sense, antioxidants are effective in reducing diabetic complications and may be beneficial either by ingestion of natural antioxidants (Bajaj & Khan, 2012).

Several tropical fruits have been reported in literature by their functional activities. Camu-camu (*Myrciaria dubia* McVaugh) is native to the Amazon region, and is considered the richest natural source of vitamin C in Brazil (Cunha-Santos et al., 2019; Chirinos et al., 2010), followed by acerola (*Malpighia emarginata D.C.*), a fruit recognized by their antioxidant properties due the phenolic and vitamin C composition (Belwal et al., 2018; Rosso & Mercadante, 2007). Cashew-apple (*Anacardium occidentale* L.) and yellow

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[1] *Embrapa Agroindústria Tropical, Fortaleza, CE, Brasil*
[2] *Faculdade de Nutrição, Universidade Federal de Alfenas – UNIFAL, Alfenas, MG, Brasil*
[3] *Departamento de Tecnologia de Alimentos, Universidade Federal do Ceará – UFC, Fortaleza, CE, Brasil*
[4] *Universidade da Integração Internacional da Lusofonia Afro-Brasileira – UNILAB, Redenção, CE, Brasil*
*Corresponding author: ana.dionisio@embrapa.br

mombim (*Spondias mombim* L.) belongs to the Anacardiacea family, and are important fruits in the Northeast region of Brazil, rich in bioactive compounds and responsible for a sweet and sour taste (Rufino et al., 2010). Furthermore, acai (*Euterpe oleracea* Mart.) is one of the most popular fruit from Amazon and widely used in the world due to recognized antioxidant properties (Yamaguchi et al., 2015).

Pereira et al. (2015) evaluated the synergistic, antagonistic and additive effects of different tropical fruits (camu-camu, cashew-apple, acerola, yellow mombim and acai), with aim to obtain a beverage with high levels of bioactive compounds. Furthermore, the beverage was evaluated *in vivo,* and shown to be effective in endogenous antioxidant defense of health Wistar rats, altering the catalase (CAT) and glutathione peroxidase (GPx) activities, and decreasing the lipid peroxidation in liver and serum in the animals (Pereira et al., 2014). Moreover, the antimutagenic and antiproliferative activities were also demonstrated by Carvalho-Silva et al. (2014) using health Swiss mice.

Considering the positive effects in the antioxidant status of this tropical fruit beverage in health models, and the importance of the antioxidants compounds and FOS for diabetes, the aim of this work was to evaluate the prebiotic and antioxidant effects of a beverage composed by tropical fruits (rich in antioxidant compounds) and yacon (source of FOS), in alloxan-induced diabetic rats.

2 Materials and methods

2.1 Chemical and reagents

The reagents used were potassium persulphate from Acrós Organics and ferrous sulphate from Vetec™. HPLC grade water was prepared from distilled water using a Milli-Q system (Millipore Lab., Bedford, MA, USA). All other chemicals were purchased from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada).

2.2 Tropical fruits and yacon

Camu-camu frozen pulp (*Myrciaria dubia* (H. B. K.) McVough) was purchased at the local market in Belém – Pará – Brazil. The frozen pulp of yellow mombin (*Spondias mombin* L.), acai (*Euterpe oleracea* Mart.), acerola (*Malpighia emarginata* D.C.), cashew apple (*Anacardium occidentale* L.) and pineapple (*Ananas comosus* L. Merr), were obtained in the local market in Fortaleza – Ceará – Brazil. The yacon (*Smallanthus sonchifolius*) was obtained in a local market at Fortaleza, Ceará State, Brazil. Then, yacon extracts were processed as reported by Dionisio et al. (2013). All the pulps and the yacon extract were stored at -18 °C for further use in the experimental design.

2.3 Beverage formulations

The blend formulation was obtained in a statistical planning as reported previously (Pereira et al., 2015), composed by 10% acerola, 5% acai, 5% yellow-mombim, 5% cashew apple, 5% camu-camu, 20% pineapple and 50% water. However, in the present experiment, the water was replaced by yacon extract in different concentrations to provide the prebiotic characteristic of the beverage (due to its FOS constituents). For the prebiotic beverage, a two-level (2^2) central composite design (CCD) was employed to optimize the values of sensorial acceptance (dependent variable) using the concentration of yacon (50 to 70%) and concentration of sweetener (stevia, 0.01 to 0.10%) as independent variables. All the experiments were carried out in order to have a random selection method to minimize the effect of unexplained variability in the responses due to systematic errors. The analysis of variance (ANOVA) was applied to validate the model, and the regression coefficients were then used to generate response surfaces. The $P < 0.05$ was considered to be statistically significant. All results were performed using Statistica 7.0.

2.4 Sensorial analysis

The sensory evaluation of acceptance was carried out with fifty untrained panelists (63% female, 82% aged 18-45 years), as suggested by Meilgaard et al. (2006) in the laboratory test, using nine-point structured hedonic scales (1: 'disliked extremely' to 9: 'liked extremely') (Souza et al., 2019). The order of presentation of the samples followed balanced order. This sensory test procedure was approved by the Research Ethics Committee of the Ceará State University, under protocol number 11044529-5.

2.5 Prebiotic beverage

The optimized prebiotic beverage was prepared by mixing the blend formulation (50%), yacon extract (50%) and 0.07% of stevioside. An Armfield FT74 tubular heat exchanger unit was used to pasteurize the prebiotic beverage after preparation. The beverage, placed in a feed tank, was pumped through the heat exchanger to achieve the treatment condition (85 °C for 90 s). After heating, the samples were cooled in an ice/water bath, packed, and then stored under -18 ± 2 °C until needed for chemical analyses. For the *in vivo* assay, the beverage was lyophilized immediately after thermal processing and stored at -18 ± 2 °C prior to use.

2.6 Prebiotic beverage characterization

Physico-chemical analyses

The color was performed in a Minolta Colorimeter (Model CR-400, Konica Minolta Sensing, Inc., Osaka, Japan), with results based on three color coordinates: L* (whiteness or brightness/darkness), a* (redness/greenness), and b* (yellowness/blueness). The acidity (% citric acid) was determined according to the method of Instituto Adolfo Lutz (2008), while the pH values were obtained using a pH meter (Hanna Instruments, Romania) (Association of Official Analytical Chemists, 2005). The soluble solids content (°Brix) was determined using a refractometer at 20.0 ± 0.5 °C, as recommended by Association of Official Analytical Chemists (2005). All analyses were carried out in triplicate.

Microbiological analyses

The presence of total coliform and *Escherichia coli* in the prebiotic beverage was evaluated according to the Feng et al. (2013). Mesophilic aerobic, mold and yeast counts, and the safety microbial parameters *Salmonella* spp. according to the Andrews et al. (2016). Analyses were carried out according to

the methodology described by FDA's Bacteriological Analytical Manual, and the results were expressed as colony-forming units per milliliter (cfu/mL) of product. These analyses were carried out in triplicate.

Total Antioxidant Capacity (TAC), Total Polyphenols (TP) and Fructooligosaccharides content (FOS)

The total antioxidant capacity was measured by the ABTS, FRAP and DPPH methods. For antioxidant extraction, the procedure developed by Larrauri et al. (1997) was used. The samples were extracted sequentially with 4 mL of methanol/water (50:50, v/v) at 25 °C for 1 h, centrifuged at 25 °C, 400 g for 15 min, and the supernatant was recovered. Then, 4 mL of acetone/water (70:30, v/v) were added to the residue at 25 °C, extracted for 60 min, and centrifuged at the same condition. Methanolic and acetonic extracts were combined, and added to 10 mL with distilled water.

The free radical scavenging activity was determined by the DPPH method (Brand-Williams et al., 1995); the ABTS·+ assay was based on a method developed by Miller et al. (1993) and, for the FRAP assay, the procedure described by Benzie & Strain (1996). All the methods were used with the modifications suggested by Rufino et al. (2010). The results of DPPH method were expressed as the concentration of antioxidant required to reduce the original amount of free radicals by 50% (EC_{50}) and the values were expressed as g prebiotic beverage per g of DPPH. For the ABTS and FRAP assay, the results were expressed as μM Trolox and μM Fe_2SO_4/g of prebiotic beverage, respectively. Thus, the total polyphenols (TP) was determined by the Folin-Ciocalteu method (Obanda et al., 1997) and the results were expressed as mg GAE (gallic acid equivalent)/100 g of prebiotic beverage. The fructooligosaccharides were determined as described by Horwitz et al. (2010), and the results expressed as g FOS/100 g of prebiotic beverage.

2.7 Prebiotic beverage: in vivo studies

Animals and experimental design

The present animal study was carried out following the guidelines in the use of animals for experimental purpose after due approval from the Research Ethics Committee on the use of animals at the Federal University of Alfenas/UNIFAL-MG (Protocol no. 528/2013). Male rats weighing 90 ± 5 g were kept under standard laboratory conditions of temperature (22 ± 2 °C), relative humidity (52 ± 5%), and 12 h light-dark cycle. Diet and water were provided ad libitum. For diabetes induction, overnight fasted rats received intraperitoneal injection (i.p.) of alloxan (120 mg/kg), followed by an aqueous solution of 10% sucrose for 24 h. After four days, the glycemia was measured and all rats that presented glucose levels higher than 200 mg/dL were considered positive diabetic induced. The lyophilized prebiotic beverage and saline solution (NaCl 0.9%, for the control groups) were administered daily by gavage (0.5 mL/100 g body weight) for 30 days. Rats (n = 30, six animals for group) were divided into the following groups: (1) negative control (healthy animals receiving saline solution); (2) positive control (diabetic animals receiving saline solution); (3) diabetic animals receiving 100 mg of prebiotic beverage per kg body weight; (4) diabetic animals receiving 200 mg of prebiotic beverage per kg body weight and (5) diabetic animals receiving 400 mg of prebiotic beverage per kg body weight.

Nutritional parameters such as weight gain and food consumption were collected three times a week. The blood glucose levels were measured nine times. At the end of the experiment, rats were killed, and blood was collected in heparinised tubes (20 U heparin/mL blood) and centrifuged at 1900 *g* for 10 min. The separated plasma was stored at - 80 °C until processed. An aliquot from caecal content was used for microbial analyses and the liver was perfused with saline solution (0.9% w/v), collected and immediately frozen at -80 °C.

Catalase activity

Catalase activity (CAT) was measured in liver tissue, and the methodology used is in according to Aebi (1984). Briefly, the activity was assayed at 25 °C by a method based on the disappearance of 10 mM H_2O_2. The decomposition of H_2O_2 by CAT contained in the samples a first-order kinetic and changes in absorbance were measured after addition of H_2O_2, and then at 10 s intervals, in a total of 60 s.

Lactobacilli analysis of caecal material

To measure the prebiotic effect of the beverage, one gram of caecal material was transferred into a sterile tube and mixed with 9 mL of sterile saline phosphate solution (PBS, Sigma Aldrich) and then serially diluted (from 10^{-1} to 10^{-7}) in a Rogosa Agar SL medium (Becton Dickinson, USA). Incubation was performed at 35 °C under anaerobic conditions using the anaerobic jar with Anaerocult A (Merck, Darmstadt Germany). After 48 h incubation, the number of colonies was recorded as $\log_{10}$ cfu/g of wet sample.

2.8 Statistical analyses

The results of the optimization of the beverage were analyzed using the Statistic 7.0 ($P < 0.05$). For the in vivo assays, the GraphPad Prism 4.0 for Windows (San Diego, CA, USA) was used. A one-way analysis of variance (ANOVA) and Tukey test ($P < 0.05$) were applied to the results.

3 Results and discussion

3.1 Optimization of the beverage formulation

The Table 1 shows the results obtained for the sensorial acceptance for all formulations tested. The values obtained ranged of 4.5 to 7.0, which corresponded to "dislike slightly" to "like moderately". Considering the effect of each parameter tested (sweetener or yacon extract concentration, in %), only the sweetener presented a significant effect ($P < 0.05$), in both linear and quadratic parameters.

Analysis of variance for the dependent variables indicated that the response surface model developed was adequate ($R^2 = 82.57$). The coefficient of determination (R^2) value is quite high, indicating that a high proportion of variability was

Table 1. Experimental design and results in RSM study.

Run n°	Sweetener x_1	Yacon extract x_2	Sensorial acceptance
1	-1	-1	6.1
2	+1	-1	6.3
3	-1	+1	6.0
4	+1	+1	6.6
5	-1.21	0	4.5
6	+1.21	0	6.8
7	0	-1.21	6.6
8	0	+1.21	6.8
9	0	0	6.9
10	0	0	7.0
11	0	0	6.9

Table 2. ANOVA for response surface quadratic model.

Variation source	SS	*df*	SM	$F_{calc.}$	*p-value*
Regression	4.2	2	2.1	18.9	0.00092
Residues	0.9	8	0.1		
Lack of fit	0.9	6	0.1	44.4	0.02220
Pure error	0.0	2	0.0		
Total	5.1	10			

SS = Sum of squares; *df* = Degrees of freedom; SM = Square Means; $F_{calc.}$ = F-Calculated; *p-value* = Calculated probability.

explained by the data and that the RSM model was adequate (Table 2). Although F-value was higher than Ftab (Fcalc = 18.9; Ftab = 4.46), the lack of fit, which measures the fitness of the model, resulted in a significant *F*-value. However, the pure error is approximately zero, and for this reason the Fcalc presents high value, and consequently, is higher than the Ftab (Fcalc = 44.4; Ftab = 19.33). Analysis of variance (ANOVA) was performed considering only the statistically significant ($P < 0.05$) variables, and a valid model was defined by Equation 1:

$$Sensorial\ acceptance = 6.84 + 0.51x1 - 0.59\ x1^2 \quad (1)$$

where: x1 is coded variable (sweetener, in %).

According to Figure 1, the maximal sensorial acceptance of the beverage was obtained using 0.07% of sweetener. Considering that the yacon extract did not present statistical significance ($P > 0.05$) in the range tested (50 to 70%), the cost of yacon and the legislation (that allow the use of 50% of water), the condition defined by the authors as 50% of yacon extract and 0.07% of sweetener.

Prebiotic beverage characterization

The prebiotic beverage presents 9.40 ± 0.10 °Brix; acidity of 0.69 ± 0.00 (% citric acid); and the following color coordinates: 28.16 ± 0.75 (L*), 9.52 ± 0.13 (a*) and 10.13 ± 0.37 (b*). Furthermore, the beverage presents lower value of pH (3.38 ± 0.01). This result was expected, since the beverage were composed by fruits such as camu-camu and acerola, recognized by the high ascorbic acid contents (Cunha-Santos et al., 2019; Akter et al., 2011; Mezadri et al., 2008).

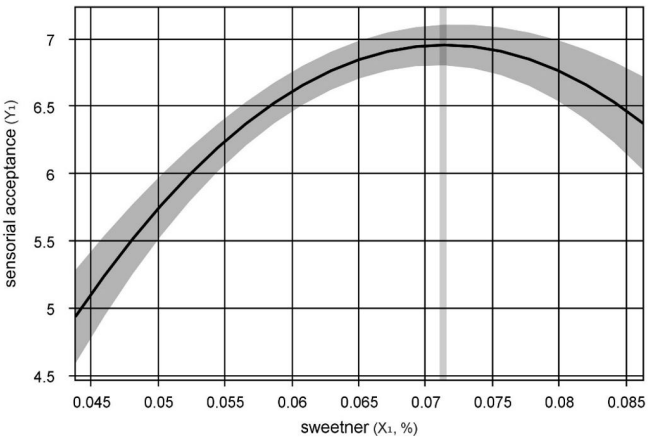

Figure 1. Effect of the sweetener concentration (%) in the sensorial acceptance of the prebiotic beverage, using 50% of yacon extract.

The total phenolic compounds (126.83 ± 9.48 mg GAE/100 g) and ascorbic acid contents (171.64 ± 7.31 mg/100 g) were also determined in the beverage. These values are in accordance with the reports of Pereira et al. (2015), using similar composition of tropical fruits, however, without yacon. The values obtained by the authors were 103.01 ± 5.96 mg GAE/100 g and 155.46 ± 2.07 mg/100 g for polyphenols and ascorbic acid, respectively. In addition, the total antioxidant activity of the prebiotic beverage were 10.57 ± 0.50 µM Trolox; 33.45 ± 0.41 µM Fe_2SO_4 and 866.36± 25.30/g of prebiotic beverage for ABTS, FRAP and DPPH method, respectively. These values are considerable high, and show the antioxidant potential of the beverage, which contributed to their functional properties.

As mentioned before, the yacon were added into beverage due the high FOS content. Considering this, the FOS was measured in the product. The values obtained were 4.64 ± 0.13 of FOS/200 mL that represents a portion of beverage. This value is about two times higher than that required by Brazilian claim for products with functional properties (Brasil, 2019), considering a minimum of 2.5 g of FOS for portion of a prebiotic beverage. In this case, the permitted claim is "FOS as prebiotics contribute to a balance/equilibrium of the intestinal flora. Their consumption should be associated with a balanced diet and a healthy life-style".

3.2 In vivo study

Non-significant differences ($P > 0.05$) among the groups that received the prebiotic beverages and their effects on diet consumption when compared to positive control (diabetic animals who received saline solution) are presented in Figure 2. Besides the diet consumption of all diabetic animals was higher than the health animals (negative control), there are no statistical difference ($P > 0.05$) in the weight gain in all treatments studied (see Figure 2).

Several papers report the potential of yacon in the glycemia control for diabetic animals or humans (Park et al., 2009; Aybar et al., 2001; Dionisio et al., 2015; Kellow et al., 2014). Unfortunately, the beverage showed no statistical effect ($P > 0.05$)

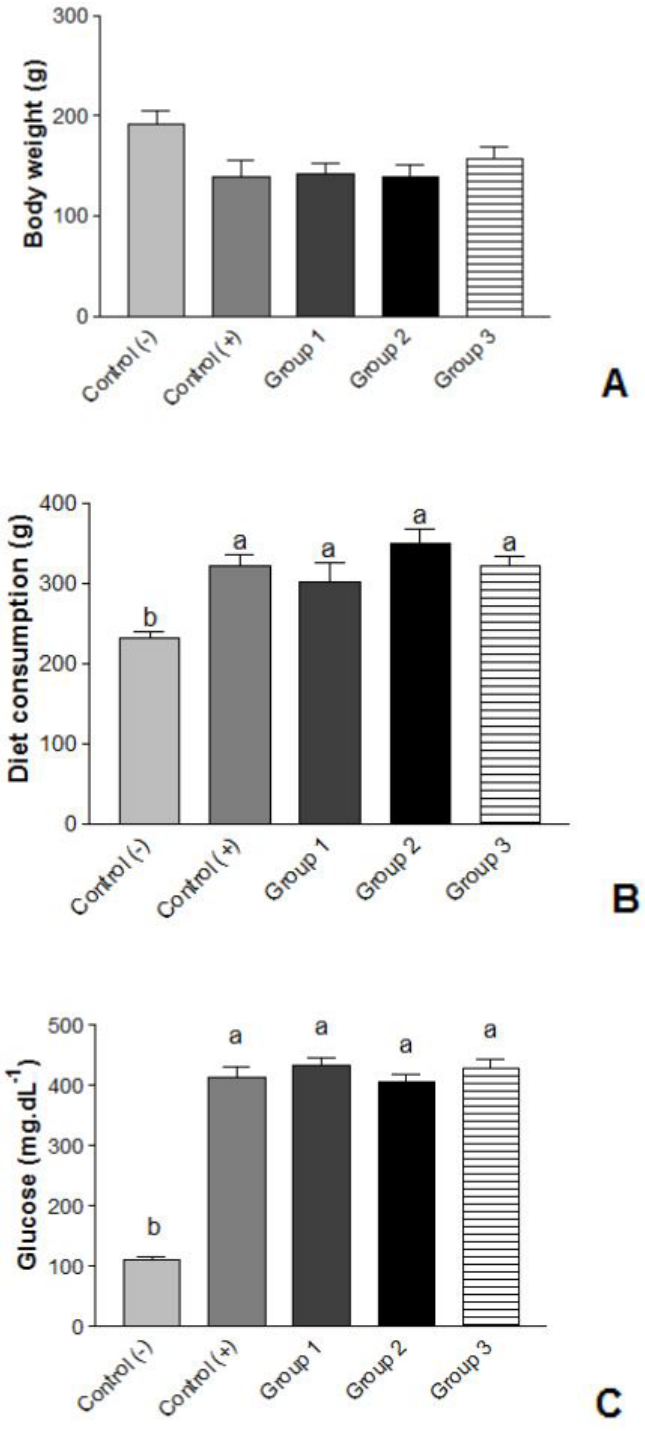

Figure 2. Effect of 30 day administration of prebiotic beverage on alloxan-induced diabetic rat on the following parameters (A) body weight (g); (B) diet consumption (g) and (C) glucose (mg.dL^{-1}).

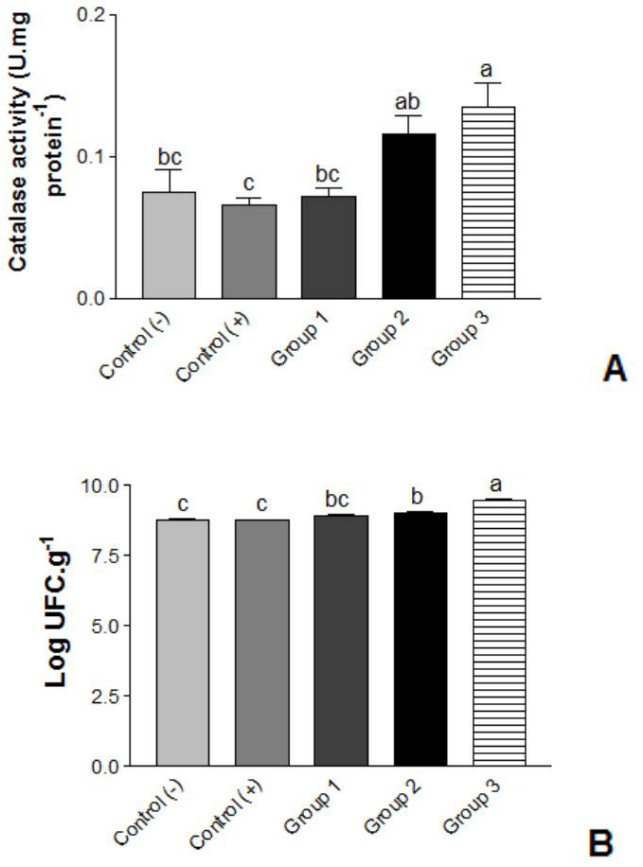

Figure 3. Effect of 30 day administration of prebiotic beverage on alloxan-induced diabetic rat on the following parameters (A) catalase activity (U. mg protein^{-1}) and (B) lactobacilli of ceacal material (log UFC .g^{-1} of wet sample).

on the glycemia of the diabetic animals in all concentrations tested (see Figure 3).

In the present experiment, was observed a decrease, although with no statistical significance ($P > 0.05$), in the catalase activity (CAT) in the liver of diabetic and non-diabetic animals. Moreover, the prebiotic beverage diet for 30 days caused a significant increase in the antioxidant enzyme activity in a dose-dependent manner. As mentioned before, the hyperglycemia is the source of most of the deleterious effects usually associated with Diabetes mellitus, and there is considerable evidence suggesting that chronic elevation of blood glucose concentrations promote auto-oxidation of glucose, and consequently leads to the generation of reactive oxygen species (ROS) (Karunakaran & Park, 2013; Ceretta et al., 2012). ROS scavenging abilities of endogenous antioxidant defenses – such as catalase, that can be can compensate by exogenous antioxidants (Bajaj & Khan, 2012). Considering this, we suggested prebiotic beverage antioxidants (phenolic, ascorbic acid and others) can protect the body against free radical-induced damage, being effective in reducing diabetic complications, as mentioned by Bajaj & Khan (2012) and Karunakaran & Park (2013).

The results from bacteriological analysis of caecal material are presented in Figure 3. Concentrations (log cfu/ g wet sample) of lactobacilli were significantly higher ($P < 0.05$) in samples obtained from animals treated with the prebiotic beverage compared to the positive (diabetic animals) or negative (health animals) control groups, in a dose-dependent manner. The prebiotic effect of yacon are supported by previous studies, showing that FOS from yacon is efficiently metabolized by lactobacilli *in vitro*

(Sousa et al., 2015a; Pedreschi et al., 2003) and *in vivo* using a Sprague-Dawley rats (Campos et al., 2012) and alloxan-induced diabetic Wistar rats (Dionisio et al., 2015).

4 Conclusion

In conclusion, the present investigation showed that the prebiotic beverage of yacon and tropical fruits possess considerable concentration of fructooligosaccharides and antioxidant compounds, that could promoted the increase the catalase activity on liver and growth of lactobacilli in the caecal material of alloxan-induced diabetic rats.

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