University of São Paulo
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Agro-industrial residues from Amazonian fruits: bioactive composition, bioaccessibility and potential use as a prebiotic

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Thesis presented for obtaining the title of Doctor in Science. Area: Food Science and Technology

Piracicaba
2023

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Bacharel in Food Science

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

De Souza Silva, Anna Paula

Agro-industrial residues from Amazonian fruits: bioactive composition, bioaccessibility and potential use as a prebiotic / de Souza Silva. - - Piracicaba, 2023.

71 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Fruits by-products 2. Phenolic compounds 3. *In vitro* gastrointestinal digestion 4. LC-MS/MS. I.Título

RESUMO

Resíduos agroindustriais de frutos amazônicos: composição bioativa, bioacessibilidade e potencial de uso como prebiótico

A intensa geração global de resíduos e subprodutos resultantes das atividades agroindustriais sinaliza a necessidade de estudos para reaproveitamento destes materiais. Este trabalho teve como objetivo determinar a composição nutricional, bioativa e o potencial prebiótico de subprodutos (tortas) do processamento do açaí (*Euterpe oleracea* Mart) e do inajá (*Maximiliana maripa* Aubl Drude) como fontes naturais de nutrientes e compostos com atividades antioxidante e anti-inflamatória. As tortas de açaí e inajá foram provenientes do processamento dos frutos para extração de óleo. Inicialmente, foram determinados a composição centesimal e o perfil mineral dos materiais brutos, seguido do potencial prebiótico utilizando bactérias probióticas (*Lacticaseibacillus rhamnosus* e *Bifidobacterium animalis* subsp. lactis). Posteriormente, os extratos das tortas foram submetidos à digestão gastrointestinal *in vitro,* obtendo-se a fração bioacessível (BF) de cada um, as quais foram analisadas por LC-ESI-QTOF-MS/MS para identificação de moléculas bioativas. Os extratos não digeridos (NDE) e suas respectivas BF foram avaliados *in vitro* quanto ao teor de compostos fenólicos totais (TPC) e capacidade de sequestro dos radicais peroxila, ânion superóxido e ácido hipocloroso. A atividade anti-inflamatória dos NDE e BF também foi determinada por meio da inibição da ativação do NF-κB e TNF-α. Os NDE e BF de açaí e inajá ainda foram avaliados quanto à toxicidade aguda em *Galleria mellonella.* Na composição centesimal, o teor de fibras alimentares foi de 80,11 ± 2,55 e 62,16 ± 3,39% para as tortas de açaí e inajá, respectivamente). Ambos os subprodutos apresentaram um perfil mineral diverso e, de modo geral, com maiores teores em comparação às polpas dos respectivos frutos. Mg, K e S estão entre os elementos presentes nas maiores concentrações. Quanto ao potencial prebiótico, ambas as tortas aumentaram a viabilidade das bactérias após 24 h de fermentação em meios de cultura contendo os subprodutos, com destaque para o resultado da torta de inajá para *L. rhamnosus* (8,3±0,1 log CFU/mL). Ácidos fenólicos, isoorientina e vitexina foram identificados na BF de açaí, enquanto os ácidos caféico hexosídeo e cítrico, glabridina e eriodictiol 7-*O*-glicosídeo foram identificados na BF de inajá, evidenciando que esses compostos possam ser responsáveis pelas atividades biológicas determinadas. Para o TPC, não foi observada diferença estatística entre o NDE e a BF de um mesmo subproduto, porém houve diferença significativa NDE e BF de subprodutos diferentes. A capacidade de sequestro do radical peroxila do NDE de açaí diminuiu cerca de 2,3 vezes após a digestão, enquanto que para o inajá não houve diferença estatística, sendo a BF de inajá 1,42 vezes mais eficiente em comparação à BF de açaí. Os NDE e as BF dos subprodutos ainda demonstraram capacidade de sequestro do ácido hipocloroso. Para a atividade anti-inflamatória, macrófagos tratados com 100 µg/mL de NDE ou BF de açaí reduziram a ativação de NF-κB em 80 e 85%, contra 33 e 96% de NDE e BF de inajá, respectivamente. Os níveis de TNF-α foram reduzidos a 70 e 81% para NDE e BF de açaí, respectivamente, e em 93% para BF de inajá. Finalmente, a avaliação de toxicidade *in vivo* indicou toxicidade nas larvas de *G. mellonella* tratadas com NDE de açaí (0,1 g/kg) e de inajá (4 g/kg). Entretanto, as BF de ambos os subprodutos não apresentaram toxicidade. Portanto, os resultados deste trabalho indicam que as tortas de açaí e inajá possuem potencial para reaproveitamento industrial como possíveis fontes de fibras e minerais com potencial atividade prebiótica; bem como para matérias-primas para a extração de compostos naturais com ação antioxidante e anti-inflamatória.

Palavras-chave: Subprodutos de frutas; Compostos fenólicos; Digestão gastrointestinal *in vitro* LC-MS/MS

ABSTRACT

Agro-industrial residues from Amazonian fruits: bioactive composition, bioaccessibility and potential use as a prebiotic

The intense global generation of waste and by-products resulting from agro-industrial activities indicates the need for studies to reuse these materials. This work aimed to determine the nutritional and bioactive composition, and prebiotic potential of by-products (meals) from the processing of açaí (*Euterpe oleracea* Mart) and inajá (*Maximiliana maripa* Aubl Drude) as natural sources of nutrients and compounds with antioxidant and anti-inflammatory activities. Açaí and inajá meals were prevenient from the processing of the fruits for oil extraction. Initially, the proximate composition and the mineral profile of the raw materials were determined, followed by the assessment of the prebiotic potential using probiotic bacteria (*Lacticaseibacillus rhamnosus* and *Bifidobacterium animalis* subsp. lactis). Subsequently, the extracts of the meals were subjected to *in vitro* gastrointestinal digestion, thus obtaining the bioaccessible fraction (BF) of each one, which was analyzed by LC-ESI-QTOF-MS/MS for identifying bioactive molecules. The non-digested extracts (NDE) and their respective BF were evaluated *in vitro* for the total phenolic content (TPC) and scavenging capacity against the radicals peroxyl, superoxide anion, and hypochlorous acid. The anti-inflammatory activity of NDE and BF was determined by the inhibition of NF-κB activation and TNF-α. The NDE and BF of açaí and inajá were also evaluated for acute toxicity in *Galleria mellonella*. In the proximate composition, the dietary fiber content was 80.11 ± 2.55 and 62.16 ± 3.39% for açaí and inajá, respectively). Both by-products showed a diversified mineral profile and, in general, with higher levels compared to the pulps of the respective fruits. Mg, K and S are among the elements present in higher concentrations. For the prebiotic potential, both meals increased the viability of the bacteria after 24 hours of fermentation in culture media containing the by-products, with emphasis on the result of the inajá meal for *L. rhamnosus* (8.3±0.1 log CFU/mL). Phenolic acids, isoorientin, and vitexin, were identified in açaí BF, while caffeic acid hexoside, citric acid, glabridin, and eriodictyol 7-O-glucoside were identified in inajá BF, attesting that these compounds may be responsible for the assessed biological activities. For TPC, no statistical difference was observed between NDE and BF of the same by-product, but a significant difference was observed between NDE and BF of different by-products. The peroxyl radical scavenging capacity of açaí NDE decreased about 2.3 times after digestion, while for inajá there was no statistical difference, with inajá BF being 1.42 times more efficient compared to açaí BF. The NDE and BF of the by-products also were capable of scavenging hypochlorous acid. For anti-inflammatory activity, macrophages treated with 100 µg/mL of NDE or BF from açaí reduced NF-κB activation by 80 and 85%, against 33 and 96% of NDE and BF from inajá, respectively. TNF-α levels were reduced by 70 and 81% for açaí NDE and BF, respectively, and by 93% for inajá BF. Finally, the evaluation of *in vivo* toxicity indicated toxic effects in *G. mellonella* larvae treated with NDE of açaí (0.1 g/kg) and inajá (4 g/kg). However, the BF of both by-products did not present toxicity. Therefore, the results of this work indicate that açaí and inajá meals have the potential for industrial reuse as possible sources of fibers and minerals with potential prebiotic activity; as well as raw materials for the extraction of natural compounds with antioxidant and anti-inflammatory action.

Keywords: Fruits by-products; Phenolic compounds; *In vitro* gastrointestinal digestion; LC-MS/MS

1. INTRODUCTION

The agribusiness activities represent important players in the Brazilian gross national product (GNP), and contributed with 26.6% (CNA, 2021), 27.5% and 25.5% in the years 2020, 2021 and 2022, respectively (CNA, 2022). Fruits in general are agricultural products with great importance in Brazilian exportations, which achieved 405.6 thousand tons and summed US$ 381.2 million in 2022 (BRASIL, 2022). Brazil has a rich plant biodiversity representing 18% of the world (Biazotto et al., 2019). Regarding the fruitful species, 220 of the 500 varieties are native to the Amazonian region and represent 44% of the total diversity of fruits in Brazil (Neves et al., 2015).

However, agro-industrial activities also generate large amounts of solid residues or by-products which have become an issue of increasing concern worldwide due to the hard management mainly with respect to their disposal (Fierascu et al., 2020). Fruit processing, for example, can generate up to 65-80% of by-products by weight of the raw material (Melo et al., 2021, Santo et al., 2012), more than 50% of the fresh fruit (Torres-Leon et al., 2018), around 60% of harvested plants (Kodagoda and Marapana, 2017), and up to 35% of the raw mass in the form of pomace (Majerska et al., 2019). Peels, seeds, pomace, husks, stems, brans, oilseed cakes, and meals are examples of the diverse by-products generated in the processing of fruits (Carrillo et al., 2022). Most of these materials is discarded as residues in the environment, thus causing contamination and pollution of water and soils. Moreover, this low sustainable process also promotes logistic and transportation problems, thus leading to environmental and economic losses (Yi et al., 2009). Therefore, fruit by-products have been investigated for the recovery of components and for potential industrial applications (Carrillo et al., 2022), such as phenolic compounds.

Phenolic compounds are secondary metabolites of plant species with one or more hydroxyl groups attached to the aromatic rings that are broadly distributed in nature and, therefore, significantly present in the human diet (de Camargo and da Silva Lima, 2019, Heleno et al., 2015). These substances are produced by the secondary metabolism of plants throughout their development and in response to stress conditions and show a range of biological properties, such as antioxidant, anti-inflammatory, antimicrobial and cardioprotective effects, to name a few (Shahidi et al., 2019). Phenolic compounds can be found either in glycosylated or aglycone forms and to date, more than 8,000 polyphenols have been investigated in detail (Ramos, 2007, De Camargo et al., 2018).

Phenolic compounds can be grouped into flavonoids and non-flavonoids, based on the structure of the aglycone forms. The flavonoids comprise a wide range of compounds, classified as flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavan-3-ols, while non-flavonoids are classified as phenolic acids, hydroxycinnamates, hydrolyzable tannins and stilbenes. The structure of the phenolic compounds can influence their biological activities and bioavailability (Eseberri et al., 2022), which can be defined as the portion of the bioaccessible compound that is absorbed and reaches the systemic circulation, thus being distributed and metabolized in the organism (Reboredo-Rodriguez et al., 2021, Velderrain-Rodriguez et al., 2014). Besides the chemical structure, the bioavailability of a phenolic compound is highly dependent on its bioaccessibility, which can be defined as the amount of a phenolic compound that is released from the matrix after the digestive process, reaching the gastrointestinal system, and that becomes available for absorption by the intestinal epithelial cells (Zahid et al., 2022, Arfaoui, 2021, Wojtunik-Kulesza et al., 2020).

The activity of phenolic compounds in scavenging reactive oxygen species (ROS), such as peroxyl radical, superoxide anion, and hypochlorous acid, in chelating metals and in reducing free radicals lead to the inhibition and/or mitigation of oxidative and inflammatory processes which are associated to several diseases (Circu and Aw, 2010, Wedick et al., 2012, Martinez et al., 2018, Romao et al., 2020, Li et al., 2020). Therefore, the ingestion of bioactive compounds from the diet is also necessary and of great relevance, because it consists of additional protection for maintaining the balance of the redox state (Pandey and Rizvi, 2009). Moreover, phenolic compounds can be industrially used as antioxidant agents, acting in food preservation, as well as in pharmacological and cosmetic products, acting as stabilizers, and inhibitors of lipid peroxidation (Luther et al., 2007, Melo et al., 2016). Brazilian native fruits are reported to present interesting antioxidant and anti-inflammatory properties mainly related to the presence of phenolic compounds (Infante et al., 2016, Lazarini et al., 2018, Lazarini et al., 2020). However, there are few studies investigating the biological activities of residues generated from Brazilian fruit processing for pulp and/or oil extraction.

Açaí (*Euterpe oleracea* Mart.), also known as açaí-do-Pará, and inajá (*Maximiliana maripa* Aubl. Drude) are species belonging to *Arecaceae* family found mainly in Pará, Amazonas, Acre, and Rondônia states, that are generally processed for obtaining pulp and oil, mainly for local and regional consumption. Nevertheless, the national and international demand for oleaginous fruits is growing due to the possibility of their use as ingredients, both in food products and in cosmetics and pharmaceuticals (Pacheco-Palencia et al., 2008, Rufino et al., 2011).

Açaí is a tropical fruit very popular in Brazil and worldwide, which has bees reported as a superfruit due to its nutritional composition and bioactive compounds with valuable properties, such as antioxidant and anti-inflammatory (Kang et al., 2011, Yamaguchi et al., 2015). Açaí fruits are small and berry-like, clustered into bunches (Rufino et al., 2011) with higher fructification between July and September (Shanley et al., 2005). The market of this fruit shows a growing demand which is superior to the offer of the fruit, both locally in the producing regions, as national (Brazilian market), or internationally, which has impacted the prices and increased the exportation of this fruit (CONAB, 2022). Brazil is the biggest exporter of frozen açaí pulp, and Pará state is highlighted as the major producer of açaí in Brazil. In 2021, the Brazilian production of açaí reached nearly 1.5 million tons (Statista, 2022b, IBGE, 2021), with Pará state leading and representing 93.5% of the total amount of the fruits produced (Statista, 2022a). The main product extracted from the fruits is the pulp while the seeds, removed for pulp or oil extraction, represent 85-95% of the total volume of the fruit (Pompeu et al., 2009). For oil extraction, açaí fruits are generally macerated in hot water for removing the seeds. Afterward, the remaining fraction is dried and mechanically pressed for extracting the oil (de Souza Silva et al., 2023), thus yielding 50% of the oil depending on the processing conditions (Yamaguchi et al., 2015), and large amounts of a fiber-rich by-product are generated at the end of the process, named meal, which has been recently investigated, for the first time, for the phenolic composition as well as for antioxidant, anti-inflammatory and antimicrobial activities (de Souza Silva et al., 2021, de Souza Silva et al., 2023).

Inajá (*Maximiliana maripa* Aubl. Drude) is the fruit of inajazeiro palm tree, which fructifies between January and March in Pará state, and at the beginning of November in Acre state. Inajá pulp has around 23% (Shanley et al., 2012) to 35.50% oil, showing a potential to be used as a raw material in the cosmetic, soap, and food industries (Bezerra, 2011). For mechanical oil extraction, inajá fruits are vapor heated and mashed for removing the pulp. Then, the pulp is heated up to 90 °C and pressed for extracting the oil (Shanley et al., 2012), when large amounts of inajá meal are generated since the yield in oil is low. This fruit still has not an added value or a large market such as açaí, being mostly consumed in the producing regions. However, this species has received attention and becoming the target of research regarding its use as a biofuel (Stachiw et al., 2016), which can even benefit the communities in which the palm tree is present, with the generation of employment, incomes, and energy for isolated areas (EMBRAPA, 2014).

Since plant products are natural sources of phenolic compounds, new investigations have been developed with plant processing by-products aiming to discover new alternative sources of bioactive substances from these materials generated in large amounts (Melo et al., 2021, Melo et al., 2020, de Camargo et al., 2016, Oldoni et al., 2016, Melo et al., 2015). However, more studies of the properties of residues generated in food processing, such as meals, are needed considering the bioactive potential of these materials, as well as their great potential to be incorporated as ingredients in several applications, such as cosmetics, pharmaceutical, with a perspective for incorporation in the human feed once they present high nutritional value, mainly in minerals and fibers (Becker et al., 2018).

Agro-industrial by-products may generate fiber-rich ingredients with functional and technological properties (Selani et al., 2014). Besides nourishing by providing proteins, lipids, carbohydrates, and other non-nutritional compounds, such as antioxidants (Torres-Leon et al., 2018), the composition of açaí and inajá meals in macro and micronutrients can also indicate potential prebiotic effects, thus benefiting the development of probiotic bacteria and increase their population in the colon. Probiotics can be defined as live micro-organisms that confer health benefits to the host when administered in adequate amounts (Hill et al., 2014), while prebiotics can be defined as a substrate that is selectively utilized by host microorganisms conferring health benefits (Gibson et al., 2017).

Açaí and inajá meals are still underexplored by the scientific community. Recently, both by-products were primarily studied for an initial characterization of biological activities and phenolic profile of their respective hydroethanolic extracts (de Souza Silva et al., 2021, de Souza Silva et al., 2023). However, to the best of our knowledge, no studies have been performed for assessing the proximate composition and mineral profile of these materials to date, which can be related to a prebiotic potential. Also, the behavior of the *in vitro* activities, as well as the profile in biaoctive compounds of the extracts subjected to *in vitro* gastrointestinal digestion, were unclear and non-investigated to date.

Therefore, the objective of this investigation was to characterize the by-products generated in the processing of açaí and inajá fruits to act as potential sources of dietary fibers and minerals, also investigating for the first time the prebiotic potential of those by-products on the counting of probiotic bacteria strains; and to assess the phenolic profile, antioxidant and anti-inflammatory capacities and *in vivo* toxic effects of the bioaccessible fractions of açaí and inajá meals.

For this, this work was divided into four work packages, which originated the two scientific manuscripts of this thesis (starting on page 27 and on page 46, respectively). The first manuscript assesses the proximate composition, mineral profile, and the prebiotic potential of açaí and inajá meals is presented. The second comprises the study investigating the phenolic profile of açaí and inajá meals after *in vitro* simulated gastrointestinal digestion, the antioxidant and anti-inflammatory activities of both non-digested and digested extracts, as well as the investigation for acute systemic toxicity of the two forms of the extracts using an *in vivo* model of *Galleria mellonella*. Hence, our findings contribute to presenting new possibilities for the use and valorization of agro-industrial by-products generated from Brazilian native fruits.

2. CONCLUSION

This study demonstrated that both by-products from the processing of açaí and inajá meals for oil extraction are rich sources of total dietary fibers, mainly the insoluble ones. Additionally, the meals presented a diverse profile of macro and microminerals, which are of great importance for the human diet, and that are present in higher concentrations compared to the pulps of the respective fruits. The prebiotic potential of both by-products was indicated by the increase in the viability of probiotic bacteria strains (*L. rhamnosus* and *B. animalis* subsp. Lactis) after 24 h of incubation. This finding may be mainly related to the total dietary fiber content of both by-products. Moreover, the samples did not exert antimicrobial effects at the established conditions. Therefore, açaí and inajá meals could be considered new natural sources of dietary fibers and minerals to be used as new food ingredients, with potential prebiotic activity. This study investigated the raw materials and opens new opportunities for further studies to explore the same parameters for the extracts produced from the by-products, also considering other probiotic strains grown individually or in co-cultures. Moreover, studies assessing the influence of the fermentation of the by-products by probiotic strains on the proximate composition, as well as determining the produced metabolites, are encouraged. Furthermore, the evaluation of different lots from the same and different regions would be important for developing an initial characterization standard of identity and quality of these materials for each region, which nutritional composition could be related to the response of the probiotic micro-organisms.

The results of this study indicate that antioxidant and anti-inflammatory activities are to some extent preserved after gastrointestinal digestion. Even gastrointestinal digestion affects the antioxidant capacity of the digested fractions somehow, the results are higher when compared to extracts obtained from other natural products. Furthermore, the bioaccessible fractions of both meals showed an important reduction of inflammation biomarkers by inhibiting the activation of NF-κB and decreasing TNF-α levels. This suggests that the remaining phenolic compounds in BF of both meals may still have an important role in the modulation of inflammatory processes when released from the matrix. Also, the bioaccessible fractions of each sample did not promote *in vivo* acute systemic toxicity, thus being safe at the same time while still conserve biological activities. Therefore, açaí and inajá meals can be considered important sources of natural bioactive compounds that may reach the small intestine for the development of new functional foods, or to be used as bioactive ingredients in food, cosmetic or pharmaceutical industries. Future studies may be developed employing dynamic digestion models for better simulating *in vivo* digestion process together the co-culture of cells for evaluating the permeability and the uptake of phenolic compounds, so that their bioavailability could be estimated closer to real metabolism conditions, also analyzing the effects of this new approach in the antioxidant, anti-inflammatory and toxicological responses.

ACKNOWLEDGEMENTS: The authors acknowledge the Sao Paulo Research Foundation (FAPESP, grant #2019/14358-0) and the Coordination for the Improvement of Higher Education Personnel (CAPES, finance code 001) for scholarships and financial support.

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