AMANDA LAIS ALVES ALMEIDA NASCIMENTO

STUDY OF STRATEGIES TO IMPROVE THE BIOAVAILABILITY OF ANTHOCYANINS

Thesis submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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To “Dona Celina”
dedicated.

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“So far the LORD has helped us.”
(Samuel 7_{12})

ABSTRACT

NASCIMENTO, Amanda Lais Alves Almeida Nascimento, D.Sc., Universidade Federal de Viçosa, March, 2024. **Study of strategies to improve the bioavailability of anthocyanins**. Adviser: Paulo Cesar Stringheta. Co-advisers: Monique Renon Eller and Evandro Martins.

Anthocyanins are phenolic compounds present in many natural products and known for their health benefits. These benefits are directly related to their bioaccessibility, which is, the ability of those substances to reach the intestine after the digestive process, being absorbed and used by the organism. In this context, this thesis aims to investigate strategies to increase the bioaccessibility of anthocyanins for human consumption. The first article consists of a comprehensive review of technological tools and strategies to improve the stability, bioaccessibility and bioavailability of anthocyanins in food systems. Additionally, we highlight methodologies for evaluating their bioaccessibility and bioavailability, which vary between in vitro and in vivo approaches. We observed that in vitro methodologies are the most widely used due to their relative financial accessibility, lower complexity, and ability to provide rapid results. Among the most used strategies to increase the stability of anthocyanins during the digestion process, microencapsulation stands out. This technique proved to be effective in protecting against pH variations and enzymatic action, resulting in a general improvement in the in vitro bioaccessibility. The review also addresses the influence of the food matrix and types of processing over those parameters, indicating that these strategies can have positive or negative impacts on bioaccessibility and bioavailability. Therefore, more studies are needed to delve on how such strategies, as fermentation, for example, effectively assist in the formulation of functional foods. Thus, in the second chapter we detailed the evaluation of three types of fermentation (spontaneous, lactic, and alcoholic) on the bioaccessibility of anthocyanins from açai. Furthermore, we evaluated the cytotoxic effect of fermented açai extracts before and after simulated gastrointestinal digestion (SD) in three cell lines. Dried açai extracts produced from spontaneous fermentation (SF) for 24 h presented greater bioaccessibility of phenolics (52.68%) and cyanidin-3 glycoside (27.01%) compared to unfermented açai. Similarly, lactic acid fermentation (LF) for 72 hours improved the bioavailability of phenolics (64.49%) and cyanidin-3-

rutinoside (20.00%). The fermented extracts did not presented cytotoxicity in the cell lines A549 (lung adenocarcinoma epithelial cells), HCT8 (human colon carcinoma) and IMR90 (normal human lung fibroblast), while extracts derived from 24-h alcoholic fermentation presented antioxidant and antimutagenic effects in vitro, reducing around 40% of chromosomal aberrations. This thesis highlights the relevance of processes such as microencapsulation and fermentation to increase the stability and bioaccessibility of anthocyanins, providing relevant information for the development of more effective functional foods.

Keywords: Açai. Phenolic compounds. Bioaccessibility. Fermentation.

RESUMO

NASCIMENTO, Amanda Lais Alves Almeida Nascimento, D.Sc., Universidade Federal de Viçosa, março de 2024. **Estudo de estratégias para melhorar a biodisponibilidade de antocianinas.** Orientador: Paulo Cesar Stringheta. Coorientadores: Monique Renon Eller e Evandro Martins.

As antocianinas são compostos fenólicos conhecidos pelos seus benefícios à saúde. No entanto, esses benefícios estão diretamente relacionados à bioacessibilidade dessas substâncias, isto é, à capacidade de chegar ao intestino de maneira viável após o processo digestivo e serem absorvidas e utilizadas pelo organismo. Nesse contexto, esta tese tem como objetivo investigar estratégias para aumentar a bioacessibilidade das antocianinas. O primeiro artigo consiste em uma revisão abrangente de ferramentas e estratégias tecnológicas para melhorar a bioacessibilidade e biodisponibilidade das antocianinas em sistemas alimentares. Adicionalmente, destacamos as metodologias para avaliar a bioacessibilidade e biodisponibilidade de compostos, que variam entre abordagens *in vitro* e *in vivo*. Observamos que as metodologias *in vitro* são as mais amplamente utilizadas devido à sua relativa acessibilidade financeira, menor complexidade e capacidade de fornecer resultados rápidos. Dentre as estratégias mais empregadas para aumentar a estabilidade das antocianinas durante o processo de digestão, destaca-se a microencapsulação. Essa técnica mostrou-se eficaz na proteção durante as variações de pH e ação enzimática, resultando em uma melhoria geral na bioacessibilidade *in vitro*. A revisão também aborda a influência da matriz alimentar e tipos de processamento, indicando que essas estratégias podem ter impactos positivos ou negativos na bioacessibilidade e biodisponibilidade. Portanto, são necessários mais estudos para que tais estratégias possam efetivamente auxiliar na formulação de alimentos funcionais. Adicionalmente, ao observar a carência de dados sobre o efeito da fermentação na bioacessibilidade de antocianinas, conduzimos o segundo trabalho centrado na avaliação de três tipos de fermentação de açaí (espontânea, láctica e alcoólica). Além disso, utilizamos três linhagens celulares para avaliar o efeito citotóxico de extratos fermentados de açaí antes e após a simulação da digestão gastrointestinal (SD). Os resultados indicaram que a fermentação espontânea (FS) por 24 horas, seguida de secagem (SD), resultou em um produto com maior bioacessibilidade de fenólicos (52,68%) e glicosídeo

cianidina-3 (27,01%) em comparação com o açaí não fermentado. Da mesma forma, a fermentação láctica (LF) por 72 horas melhorou a biodisponibilidade de fenólicos (64,49%) e cianidina-3-rutinosídeo (20,00%). As amostras não apresentaram citotoxicidade nas linhagens celulares testadas e, além disso, a fermentação alcoólica (24 horas) demonstrou efeitos antioxidantes e antimutagênicos *in vitro*, reduzindo cerca de 40% das aberrações cromossômicas. Em resumo, esta tese destaca a importância de processos como a microencapsulação e a fermentação para aumentar a bioacessibilidade das antocianinas. Essas descobertas oferecem informações relevantes para o desenvolvimento de alimentos funcionais mais eficazes. Entretanto, ressaltamos a necessidade contínua de pesquisa com diferentes fontes e processos para otimização dos benefícios à saúde.

Palavras-chave: Açaí. Compostos fenólicos. Bioacessibilidade. Fermentação.

SUMMARY

GENERAL INTRODUCTION

Foods rich in bioactive compounds have attracted considerable attention due to their potential health benefits. In general, a high intake of foods from plant sources has been associated with a reduced risk of chronic diseases such as cardiovascular diseases, cancer, and type 2 diabetes (AUNE, 2019; ZHU et al., 2021). In this context, açaí (*Euterpe Oleracea* Mart palm tree), also known as a "super fruit", stands out due to its high antioxidant capacity due to the presence of phenolic compounds. This fruit is native from the Amazon region and widely found in northern Brazil, and has been assessed for anticarcinogenic, anti-inflammatory, and antimicrobial properties, as well as benefits in preventing dyslipidemia and atherosclerosis. Its rounded, dark purple berries have a pulp rich in anthocyanins, arousing interest both in Brazil and internationally (KANG et al., 2012; MACHADO et al., 2019; SCHAUSS et al., 2006).

Anthocyanins, which fall into the class of flavonoids, are phenolic compounds recognized for their health benefits, acting in the prevention of chronic diseases associated with oxidative stress. Recent researches show that consuming foods rich in anthocyanins is associated with a reduced risk of conditions such as cardiovascular disease, diabetes, dementia, and cancer. However, the effectiveness of these compounds is intrinsically linked to their bioaccessibility, that is, the ability to be absorbed and used by the body (HAIR; SAKAKI; CHUN, 2021; KALT et al., 2020).

The bioaccessibility of açai anthocyanins can be affected by several factors, including food processing, gastrointestinal conditions, and interactions with other components of the diet. Understanding and optimizing the bioaccessibility of these compounds therefore becomes a crucial challenge to enhance their beneficial effects on human health (GIACONIA et al., 2022; HAO et al., 2021; TAMARGO et al., 2022). This thesis aimed to study strategies to increase the bioaccessibility of açai anthocyanins, to maximize the health benefits associated with the consumption of these substances.

In the first article, we sought to understand the strategies previously described in the literature, compiling existing knowledge about the bioaccessibility of anthocyanins. In the second article, we introduce an innovative perspective by exploring aspects not previously discussed in the literature, presenting fermentation as a new strategy to improve the bioaccessibility of these compounds.

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PAPER 1

Nascimento, A. L. A. A., Borges, L. L. R., Fernandes, J. G., Freitas, V. V., Martins, E., Campelo, P. H., & Stringheta, P. C. (2023). Exploring strategies to enhance anthocyanin bioavailability and bioaccessibility in food: A literature review. Food Bioscience, 56, 103388. https://doi.org/10.1016/J.FBIO.2023.103388

EXPLORING STRATEGIES TO ENHANCE ANTHOCYANIN BIOAVAILABILITY AND BIOACCESSIBILITY IN FOOD: A LITERATURE REVIEW

Abstract

Anthocyanins possess significant potential in improving consumer health; however, their unstable nature poses challenges to their incorporation into functional foods. This comprehensive review aims to explore various technological tools and strategies that can be employed to improve the bioaccessibility and bioavailability of anthocyanins in food systems. Low bioaccessibility refers to the limited number of anthocyanins available for absorption in the gastrointestinal tract, while low bioavailability represents the fraction that actually reaches systemic circulation. To overcome these limitations, technological tools can be utilized to improve the release and absorption of anthocyanins during digestion. Microencapsulation techniques have shown promising results in safeguarding anthocyanins from enzymatic degradation and pH variations in the gastrointestinal tract, thereby enhancing their bioaccessibility. With further research and development in this area, we can pave the way for formulating functional foods with optimized anthocyanin content and enhanced bioactive properties.

Keywords: Food anthocyanins; Bioactive compounds; Food systems; Encapsulation techniques.

1. INTRODUCTION

According to Mintel's (2023) global food and beverage projections, consumers will tend to seek products that support mental performance in daily activities such as foods and beverages with energizing plant-based ingredients with nootropic properties. Within this perspective, anthocyanins are natural pigments of the

flavonoid class with antioxidant capacity (ARANHA et al., 2019; CELLI et al., 2019; WICZKOWSKI; SZAWARA-NOWAK; TOPOLSKA, 2013) that help brain function by modulating neuroinflammation, neurogenesis and neuronal signaling (Krikorian et al., 2011, 2019; Li et al., 2021; Zhang et al., 2020). In a study carried out by Gibson et al., (2020) with Rugby players, it was concluded that the consumption of a nootropic drink based on blackcurrant rich in anthocyanins, improved the perception of confidence and reduced the distraction of sportsmen. In another study, Micek et al. (2022) verified that the increase in the consumption of fruits rich in anthocyanins such as strawberries, cherries, prickly pears and grapes reduced the occurrence of poor sleep quality, perception of stress and depressive symptoms.

Scientific evidence of the neurological benefits associated with the frequent consumption of anthocyanins can serve as a starting point for including extracts from red fruits (blackberry, raspberry, açai, jabuticaba, etc.) and vegetables (red cabbage, eggplant, etc.) in industrially produced foods. In addition to improving cognitive abilities, regular consumption of anthocyanin-rich vegetables can also help control blood pressure (IGWE et al., 2017), diabetes (ALNAJJAR et al., 2019; BARIK et al., 2020; SUN et al., 2023), obesity (SANTAMARINA et al., 2023; VUGIC et al., 2020), dysbiosis (CHEN et al., 2022), and even act in cancer prevention (CHIU et al., 2015; LONG et al., 2018; MAZEWSKI; LIANG; GONZALEZ DE MEJIA, 2017; NASCIMENTO et al., 2023b; SU et al., 2018), thrombosis (THOMPSON et al., 2017), and cardiovascular diseases (BASU et al., 2010; CASSIDY et al., 2016; DE LIZ et al., 2020). However, despite the broad functionality of anthocyanins in the human body, potential consumer health benefits may be reduced due to the unstable nature of these substances.

Anthocyanins undergo accelerated degradation when exposed to light, oxygen, pH above 7, and temperatures above 50 ºC (SANTOS-BUELGA, 2018; SUI et al., 2018; SUI; DONG; ZHOU, 2014). These adverse environmental conditions are commonly encountered during food and beverage processing or storage, which poses a challenge for incorporating anthocyanin-rich plant extracts into industrial food formulations. The instability of anthocyanins also affects their preservation during digestion, mainly due to the presence of enzymes and sudden pH variations, resulting in significant losses during their passage through the gastrointestinal tract (GIACONIA et al., 2022; HAO et al., 2021; TAMARGO et al., 2022).

The beneficial effects of these compounds on consumer health are directly linked to their absorption; thus, low bioaccessibility and bioavailability can limit their effectiveness. Bioaccessibility refers to the amount of a given compound that is available for absorption in the human gastrointestinal tract, while bioavailability refers to the fraction of these compounds that reaches systemic circulation (SANTOS et al., 2019). Once in circulation, the compounds can be metabolized or stored in target tissues.

Considering the high potential of these substances to be incorporated to formulation of functional foods, technological strategies must be employed to enhance the bioaccessibility and bioavailability of anthocyanins, particularly during food processing and the passage through the gastrointestinal tract (da Silva Haas et al., 2019; Dantas et al., 2021; Nascimento et al., 2023; Zou et al., 2021).

In this review, we will discuss the main technological tools that can be employed to improve the stability of anthocyanins in food systems and enhance their bioaccessibility and bioavailability. Among the strategies described, special emphasis will be placed on microencapsulation techniques due to the promising results discussed in recent scientific literature.

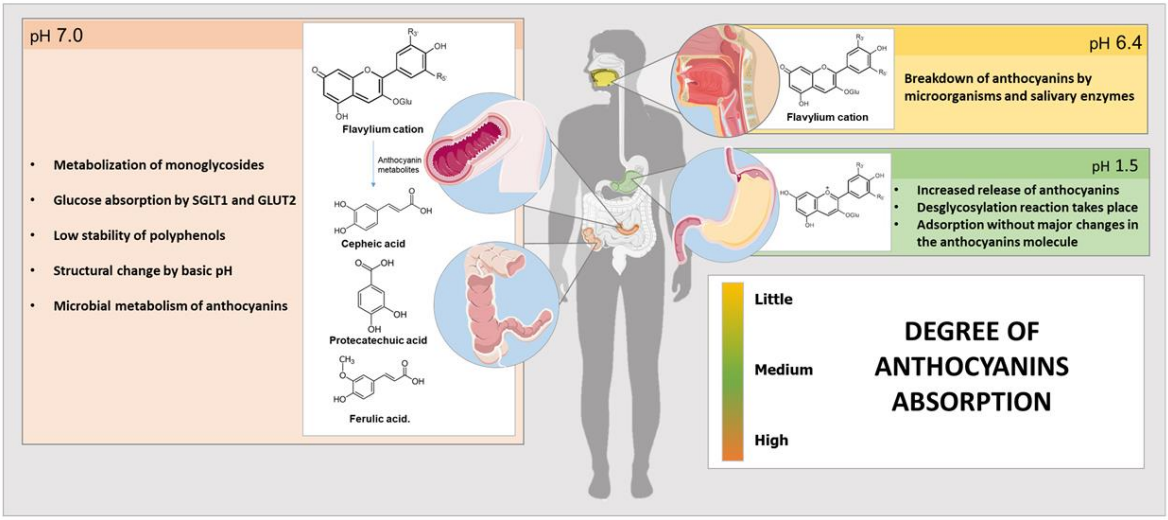
2. TOOLS TO MEASURE THE BIOACCESSIBILITY AND BIOAVAILABILITY OF ANTHOCYANINS

Digestion is a complex multifactorial process that involves everything from the mechanical grinding of food to the action of enzymes, microorganisms and pH variations. There is still no exact understanding of the effect of each of these factors during the digestion of phenolic compounds (Archivio et al., 2010; He et al., 2016); however, considering the pH variations that occur during the digestion, i.e. saliva (pH~6.4), stomach (pH~1.5) and intestine (pH~7.0), it is to be expected that anthocyanins can occur in different chemical forms (Figure 1) which affect their bioaccessibility and bioavailability in the human body.

When ingested, the food is fragmented into smaller pieces through chewing and, depending on the time it remains in the mouth, the microorganisms and enzymes present in the saliva or even in the food matrix can contribute to the release and breakdown of anthocyanins (HAN et al., 2019; MALLERY et al., 2011). In addition, mechanical rupture increases the surface area for interactions with digestive enzymes and salivary proteins (KARAŚ et al., 2017). It was demonstrated by

Kamonpatana et al., (2014) that anthocyanins can be degraded in the human oral cavity and that the loss is partially due to microbial activity. The main metabolites of anthocyanins found in the oral cavity were protocatechium acid, aldehyde floroglucinol and anthocyanin glucuronide conjugates (KAMONPATANA et al., 2012; MALLERY et al., 2011).

Figure 1. Different structures of anthocyanins during gastrointestinal digestion.



Legend: The digestive process begins with chewing, where the action of microorganisms and enzymes in saliva, together with mechanical breakdown, contributes to the release and fragmentation of anthocyanins. In the stomach, the low pH softens the food matrix and favors the release of anthocyanins, which can be absorbed in the gastric wall. Subsequently, in the small intestine, glycosylated anthocyanins can be transported and transported through glucose transporters, while enzymes such as β-glucosidase promote their transformation into more easily absorbable compounds. In the large intestine, commensal microbiota plays a crucial role in breaking down anthocyanins into metabolites that can be absorbed or excreted.

In the stomach, the low pH promotes the softening of the food matrix and helps in the release of anthocyanins. Although the stomach is not considered a place of nutrient absorption, some studies suggest that anthocyanins can permeate the gastric wall and be absorbed without major changes in the molecule structure (MARQUES PEIXOTO et al., 2016). In general, studies have shown that, after consumption, anthocyanins are quickly found in plasma, precisely because this absorption occurs in the gastric wall (FARIA et al., 2013a; TALAVÉRA et al., 2005). Corroborating this, Felgines et al. (2006) evaluated the absorption and metabolism of red orange anthocyanins in rats. In in situ analysis, it was observed that about 20%

of red orange anthocyanins were absorbed by the animal's stomach. A study conducted with humans shows that after ingestion of encapsulated anthocyanins, high urinary levels of these phenolics and their degradation products could be detected. The authors concluded that the sudden increase in anthocyanin levels in the urine is due to the adsorption of these substances through the stomach epithelium (MUELLER et al., 2018).

In the small intestine, glycosylated anthocyanins can be carried and absorbed with the aid of glucose transporters (SGLT1 and GLUT2) (Barik et al., 2020; Fernandes et al., 2017). In addition, enzymes such as β-glucosidase and lactase-chloridine-hydrolase can promote the breakdown of glycosidic bonds or the cleavage of the C ring forming phenolic acids of lower molecular weight and more easily absorbed (Ávila et al., 2009; Fernandes et al., 2014).

It is estimated that only 5 to 20% of the total intake of phenolics is absorbed in the small intestine (MUELLER et al., 2018; YUSTE et al., 2019). In this way, the remaining compounds reach the large intestine where the commensal microbiota is responsible for the decomposition of the original phenolic structures into metabolites that can be absorbed or excreted by the human body (BAO et al., 2019a; CORREA-BETANZO et al., 2014; VAN DE VELDE; PIROVANI; DRAGO, 2018). In a study conducted by Inada et al., (2020), it was found that after simulated digestion followed by fecal fermentation, the microorganisms present in the large intestine extensively metabolized the delphinidine-3-O-glycoside present in jabuticaba and it was not possible to detect it at the end of fermentation. While there was a decrease in delfinidin-3-O-glycoside, an increase in the gallic acid metabolite was also observed, indicating a bioconversion. For cyanidin-3-O-glycoside, the decrease in its content coincided with the appearance of cyanidin, suggesting that the intestinal microbiota promoted its deglycosylation.

For the evaluation of the bioavailability and bioaccessibility of anthocyanins present in food, in vitro and in vivo methodologies can be used, with human or animal models (SANTOS et al., 2019). Braga et al. (2018) cite that the need to develop in vitro methodologies was since initially in an in vivo experiment there was a very low bioavailability of anthocyanins, which led the researchers to try to simulate digestion in order to understand how these compounds behaved during the process and what were the influences of each stage of digestion.

In in vitro assays, simulated digestion is used to estimate the bioaccessibility of anthocyanins. For this, digestion models are used that mimic the physicochemical and physiological events that occur in the human gastrointestinal tract. Within this approach, some studies of simulated digestion of samples rich in anthocyanins and anthocyanins alone have shown that these compounds have high stability during the gastric phase and reduced recovery during the intestinal phase (DE MORAIS et al., 2020; QUATRIN et al., 2020; VICTORIA-CAMPOS et al., 2022). Within in vitro methodologies, dialysis and centrifugation are two commonly used techniques to estimate the bioaccessible fraction of anthocyanins. Van de Velde et al., (2018) used dialysis in the intestinal phase to provide more correct estimates of bioaccessibility. The authors analyzed the dialysable fraction (bioaccessible), which represents the compounds potentially absorbed by enterocytes during passage in the intestine, and the non-dialysable fraction that simulates the compounds that would go to the small intestine for colonic fermentation. The results showed that the bioaccessibility of cyanidin-3-glycoside, the main anthocyanin quantified in blackberry fruits, was less than 2%.

In order to simulate biological systems, in vitro assays with cell culture have been developed to study the bioavailability of anthocyanins and the mechanisms involved in the absorption of these flavonoids (KOSIŃSKA-CAGNAZZO et al., 2015). Caco-2 cells are the most used to estimate bioavailability at the intestinal level, because, under specific culture conditions, they are differentiated to form a monolayer of polarized epithelial cells that resembles the enterocytes that line the small intestine, making them ideal for simulations of intestinal absorption (LU; QI; WU, 2018). MKN-28 cells (differentiated cells of adenocarcinoma of the stomach) are used to simulate how anthocyanins are transported/absorbed in a gastric cell barrier model (MANOLESCU et al., 2019). In both methods, the bioavailability of anthocyanins was reported as low, with transport/absorption percentages of less than 5% in Caco-2 cells and ranging from 4-19 % for MKN-28 cells (CARDONA; MERTENS-TALCOTT; TALCOTT, 2015; HAN et al., 2020; KOSIŃSKA-CAGNAZZO et al., 2015; KUNTZ et al., 2015; MARQUES PEIXOTO et al., 2016; OLIVEIRA et al., 2019a).

In vitro assays have the advantage of evaluating the stability and structural changes of bioactive compounds before absorption. In addition, these techniques are relatively cheap, less laborious and allow faster results to be obtained (Alminger et

al., 2014; Gutiérrez-Grijalva et al., 2017; Guerra et al., 2012; Herrera-balandrano et al., 2021; Victoria-Campos et al., 2022). On the other hand, in vitro assays are limited regarding the effective reproduction of the complexity of the gastrointestinal tract mainly with regard to the host microbiota, peristaltic movements and the presence of enzymes (BROWN et al., 2014; GLEICHENHAGEN; SCHIEBER, 2016).

Bioavailability can be determined using in vivo models in animals or humans, by quantifying the concentration of anthocyanins and their metabolites in the blood, urine and feces after ingestion of foods or beverages rich in these compounds. In in vivo trials with animals there is still the possibility of evaluating target tissues such as heart brain, kidneys liver, etc. The bioavailability of anthocyanins in in vivo studies was estimated to be very low, with recoveries of less than 1% of the ingested anthocyanin dose (CHARRON et al., 2009; KUNTZ et al., 2015; MUELLER et al., 2017; ZHONG et al., 2017).

Despite the complexity, bureaucracy, ethical factors and the high cost of conducting tests, in vivo methods are still considered more reliable, because they occur within a living organism (BROWN et al., 2014). However, in studies of bioavailability and bioaccessibility of anthocyanins, there is still no consensus on the accuracy of the results of in vitro and in vivo tests. In this way, most research has used in vitro methodologies due to the possibility of applying unlimited numbers of samples, which allows sample screening, hypothesis formation and such techniques facilitate reproduction, standardization and methodological repeatability (BRAGA et al., 2018a; MINEKUS et al., 2014).

3. STRATEGIES TO INCREASE THE BIOAVAILABILITY AND BIOACCESSIBILITY OF ANTHOCYANINS IN FOOD

3.1. Co-ingestion and changes in the food matrix

The food matrix or co-ingested food matrices are capable of interfering with the digestion and absorption of anthocyanins (Figure 2). It was shown that the co-ingestion of pomegranate with sunflower oil, UHT skimmed milk, bread, skimmed natural yogurt, protein yogurt, red apple, lemon, honey, soybean or wheat starch did not result in a significant difference ($p<0.05$) in the total anthocyanin content during gastric conditions and for the unabsorbed fraction (bioaccessible). The unabsorbed and potentially absorbed fractions were obtained by dialysis during simulated

intestinal digestion. The content of total anthocyanins in the bioaccessible fractions were higher than the values obtained for the bioavailable fraction when the pomegranate was co-ingested with meat protein, stearic/linoleic acids, lactose, galactose fructose, starch or cellulose. That is, anthocyanins were bioaccessible, but were not absolved during the dialysis process (SENGUL; SUREK; NILUFER-ERDIL, 2014).

Figure 2. Bioaccessibility of anthocyanins co-ingested with different foods.

Legend: Effect of co-ingestion of fruit extracts rich in anthocyanins with (+signal) other food components on the bioaccessibility of anthocyanins. The upward-pointing arrow indicates an increase in bioaccessibility, while the downward-pointing arrow indicates a reduction in bioaccessibility.

Ribnicky et al. (2014) demonstrated that a lipid-rich food matrix of the type can selectively affect the bioaccessibility of blueberry anthocyanins in an intestinal cell culture model. The matrix used was a mixture of boiled eggs, bacon, whole cow's milk, butter, white bread and margarine. It was observed that delphinidin-3-glucoside, delphinidin-3-galactoside, delphinidin-3-arabinoside and petunidin-3-arabinoside were twice as bioaccessible in the presence of lipid-rich food matrix while delphinidin-3-(600-acetoyl)-glycoside and malvidin-3-arabinoside were twice as bioaccessible when blueberry was digested alone.

The co-ingestion of red cabbage with foods rich in carotenoids (baby spinach, cherry tomatoes or with a mixture of carrots, baby spinach and cherry tomatoes) increased the bioaccessibility of total anthocyanins ranging from 10-15% ($p < 0.05$). The co-digestion of red cabbage only with carrots did not affect the bioaccessibility of anthocyanins ($p < 0.05$), however the bioaccessibility of carotenoids was decreased between 21–33% depending on the carotenoid analyzed ($p < 0.05$). The authors suggest that the antioxidant activity of food matrix carotenoids may have contributed to reducing the susceptibility of anthocyanins to oxidative degradation during digestion, resulting in increased bioaccessibility (PHAN; BUCKNALL; ARCOT, 2019a). Unlike what was observed in the previous study, the addition of cabbage (food rich in carotenoids and chlorophyll) led to a decrease in the amounts of strawberry anthocyanins available during gastric digestion. However, during the intestinal phase, no significant effect was observed (STÜBLER et al., 2020).

Two different fractions of pectin were tested together with blueberry extract and anthocyanin patterns during simulated digestion. It was observed that the chelating-soluble fraction (linear, with strong negative charge) prevented the degradation of malvidine-3-glycoside, cyanidin-3-glycoside and anthocyanins of the blueberry extract during simulated gastrointestinal digestion, increasing bioaccessibility. The water-soluble fraction increased the bioaccessibility of cyanidin-3-glycoside (KOH; XU; WICKER, 2020). In contrast, in another study, the addition of pectin (5%) to red raspberry puree significantly impaired the bioaccessibility of total anthocyanins (25-30%) after in vitro digestion. Individually, pectin reduced the bioacceptibility of cyanidin-3-glycoside (14%), cyanidin-3-rutinosid (17%), pelargonidin 3-glycoside (16%) and peonidine (28%) when compared to control ($p < 0.05$) (TOMAS, 2022).

The protective effects of α-casein and β-casein on blueberry anthocyanins using an in vitro digestion model were investigated by Lang and collaborators (2021). The content of anthocyanins at the end of the simulated digestion with α-casein (0.042 mg C3G/mL) or β-casein (0.032 mg C3G/mL) was significantly higher than that of isolated anthocyanins, indicating protective effects of caseins on blueberry anthocyanins that had conserved antioxidant capacity. The protective effect of caseins on anthocyanins has been attributed to the ability to form bonds between milk proteins and these phenolic compounds (LANG et al., 2019).

In another study, excretion of anthocyanins in rats treated with bilberry extract or bilberry extract added with α-casein (LANG et al., 2023). In the presence of α-casein, the anthocyanin content decreased in feces and urine and increased in the blood, illustrating that α-casein can promote better absorption of anthocyanins, that is, greater bioavailability. It was also possible to observe an increase in the content of anthocyanin metabolites in the urine (syringic acid, ferulic acid, 4-hydroxybenzoic acid and vanillic acid) indicating that α-casein can prolong the reaction time of anthocyanins with the intestinal microbiota in vivo, increasing the absorption of metabolites in the blood and consequently in the urine by filtering the blood in the kidneys (LANG et al., 2023).

As seen, research dedicated to the subject suggests that some compounds such as carotenoids and α-casein can improve the bioavailability/bioaccessibility of anthocyanins and, thus, the incorporation of these ingredients into food systems can be the starting point for improving the absorption of phenolic compounds by the human body (LANG et al., 2023; SENGUL; SUREK; NILUFER-ERDIL, 2014). Despite this indication, the behavior of anthocyanins in relation to the other components of the food formulation may be contrary to the intended, which justifies the need for in-depth studies for each type of food with functional claim.

3.2. Changes in food processing

Anthocyanins are sensitive to pH and temperature variations, exposure to light, oxygen, metal ions, enzymes and sugars (KAMILOGLU et al., 2015; SANTOS-BUELGA, 2018; SUI et al., 2018). These adverse conditions are recurrent during the processing of various industrialized foods and this enhances the degradation of these compounds and the reduction of their bioavailability and bioaccessibility during digestion. In this way, changes in the processing of certain foods can be valid strategies to preserve higher levels of anthocyanins in these products.

During the processing of black carrots in jelly and marmalade there was a loss of 87.6% and 95.6%, respectively, of anthocyanins. However, during gastric digestion, slight increases (5.9–10.3%) in the non-acylated anthocyanin values of marmalades were detected. At the end of digestion, there was an increase in the percentage of recovery of individual bioaccessible anthocyanins from jellies (0.8 %) and marmalades (10.3%) compared to fresh carrots. That is, despite the significant

losses during the production of these sweets, it can be observed that the processing favored the bioaccessibility of the anthocyanins studied (KAMILOGLU et al., 2015).

The bioaccessibility and bioavailability of anthocyanins in red rice bran were increased with the use of solid-state (JANARNY; GUNATHILAKE, 2020). The percentage available after intestinal digestion ranged from 9.79% to 43.39%, indicating a significant increase in the anthocyanin content recovered after intestinal digestion in fermented samples compared to unfermented samples. The authors cite that this increase may be linked to the fact that extracellular fungal enzymes cause structural breaks in the bran matrix with a consequent increase in the bioaccessibility of bioactive compounds (JANARNY; GUNATHILAKE, 2020).

Recently, Amagloh et al. (2023) demonstrated that sweet potato root cooking methods can increase the bioaccessibility of anthocyanins. According to the authors, frying showed lower bioaccessibility for anthocyanins (4%) while for roots cooked in water the increase was 12% and steamed, about 15 times higher than raw roots (AMAGLOH et al., 2023). This increase in the bioaccessibility of anthocyanins can be explained by the softening of the food matrix through the application of heat, which facilitates the release of phenolic compounds.

As for the techniques considered emerging, non-thermal processing such as high pressure processing (HPP) and pulsed electric field (PEF) did not change the bioaccessibility of anthocyanins in blackberry juice (OZKAN et al., 2022). Similarly, the bioaccessibility of PEF-treated pelargonidin-3-O-malonylglycoside (in a strawberry-caught food matrix) was not altered during gastric digestion. In addition, this same matrix showed greater bioaccessibility byrgonidin-3-O-malonylglycoside for the sample treated with HPP (STÜBLER et al., 2020).

3.3. Encapsulation of anthocyanins

The low stability of anthocyanins during extraction, handling, storage, and consumption limits their application in food systems. As a result, several studies have pointed to encapsulation as a promising technique for the protection of these substances (PAN et al., 2022; ROSALES; FABI, 2022; SHARIF; KHOSHNOUDI-NIA; JAFARI, 2020). To get an idea of the importance of this tool for protecting and increasing the bioaccessibility and bioavailability of anthocyanins, in the last 5 years around 1200 research articles were published using the terms encapsulation and anthocyanins on the Science Direct platform.

Encapsulation is defined as the process by which liquid, solid or gaseous agents are adsorbed or trapped by a coating. The encapsulated material is called the active and normally makes up the core(s) of the capsule. The material that coats or adsorbs the active is known as wall material (JANGAM; MUJUMDAR; ADHIKARI, 2015; VIDAL JIMÉNEZ, 2016). In the case of encapsulation of anthocyanins, these will be active while the wall material can be composed of maltodextrin, starch and its derivatives, gums, soy lecithin, milk proteins, or the association of two or more of these materials (RIGHI DA ROSA et al., 2021). Depending on the wall materials and the encapsulation technique employed, capsules with anthocyanins can vary in size (1 and 500 μm), be mononuclear or polynuclear, soluble or insoluble in water (Table 1).

The encapsulation of anthocyanins directly influences the physicochemical properties of the capsules and thus modulates the release of the active compound during consumption. For the absorption of anthocyanins in the stomach, stability and timely release in an acidic environment are fundamental, while absorption in the small intestine requires stability in the gastric environment and release of the active in a slightly alkaline environment. This poses a significant challenge since anthocyanins degrade rapidly at pH levels close to neutrality (HERRERA-BALANDRANO et al., 2021).

This technique enables increased bioaccessibility and bioavailability of anthocyanins by isolating the active material from the external environment and protecting them from the conditions of the gastrointestinal tract. It provides controlled release that is directed to the site of absorption (DIAS; FERREIRA; BARREIRO, 2015). Through controlled release, encapsulation delays the release of anthocyanins into the medium and increases their availability over time, thus prolonging their retention in the gastrointestinal tract. The extended residence time of compounds on absorptive surfaces is generally associated with greater absorption and, consequently, higher bioavailability of the compounds (FARIA et al., 2013a; TARONE et al., 2020).

In addition to protecting anthocyanins from environmental factors that can alter their chemical structure (such as temperature, oxygen, pH, enzymes, etc.), encapsulation can also be utilized to change the physical state of these substances (from liquid to solid), as well as mask or enhance their sensory characteristics (color, flavor, and aroma) (RIGHI DA ROSA et al., 2021).

Given the significant importance of this technique in the application of anthocyanins in food systems, the following sections will provide detailed explanations of physical (spray drying, freeze-drying), physicochemical (coacervation, liposomes, niosomes, and ionic gelation), and chemical encapsulation techniques (molecular inclusion) for these substances. Furthermore, Table 2 summarizes the positive and negative points of each encapsulation technique.

Table 1 Encapsulation techniques and results on the bioaccessibility of anthocyanins

Technique	Active material	Wall materials	Main results	Reference
Spray dryer	Blueberry extract	Arabic gum and milk protein	- Particle size: 20 to 30 µm -Gum arabic microcapsules had higher anthocyanin content and higher release rates when compared to whey protein microcapsules.	(FLORES et al., 2014)
	Blueberry anthocyanins	Carboxymethyl starch (CMS) and xanthan gum (XG)	- EE (%): > 96%. - The release percentage of anthocyanins decreased with the increase of XG content in the system. - The percentage of anthocyanins released from CMS/XG (60/1)-ANS microcapsules in simulated gastric fluid was 21.24%, compared with 35.68% from CMS-ANS microcapsules.	(CAI et al., 2019)
	Blueberry extract	Maltodextrin DE20, hi-maize, inulin and gum arabic	- EE (%): 96,80 - 98,83%. - Particle size: 11.7 to 15.2 µm - The combination of encapsulating agents (5% maltodextrin DE20, 5% hi-maize, 5% inulin and 5% gum arabic) promoted an improvement in simulated bioaccessibility compared to the free extract.	(RIGHI DA ROSA et al., 2021)
	Blueberry anthocyanins	High methyl pectin (HMP) combined with whey protein isolates (WPI) or soy protein isolates (SPI)	- The EE of MBAP with WPI/HMP or SPI/HMP combination, ranged between 81.0% and 93.5%, which was higher than those with WPI or SPI alone, showing 75.1 ± 2.1% and 80.2 ± 1.9%, respectively - Particle size: 16.89 to 20.01 µm, which was slightly smaller than that with WPI/HMP combination, ranging from 20.81 to 24.54 µm.	(PAN et al., 2022)
	Blueberry anthocyanins	Micellar casein and whey protein isolate (WPI)	- EE (%): 49,73 - 59,99%. - Particle size: 7,87 to 8,78 µm - When micellar casein was used (CA samples), the low solubility and formation of oversized curds substantially hindered the release of ACNs; when whey protein was used (WA samples), the high solubility caused the formation of liquid microparticles and subsequently contributed to the slower release of ACNs.	(LIAO et al., 2021)
	Blueberry extract	Citrus pectin capsules (CPC) and whey proteins	- Encapsulation did not influence the bioavailability of total anthocyanins in humans.	(MUELLER et al., 2018).
Lyophilization	Blueberry	Carboxymethyl starch	- EE (%): > 96 %	(CAI et al., 2019)

	anthocyanins	(CMS) and xanthan gum (XG)	- Microencapsulated anthocyanins showed a controlled release in the gastric simulation (21.24% - 35.68%). - The % of anthocyanins released in the simulated intestinal fluid was greater than 70% after 120 minutes.	
	Maqui juice anthocyanins	Maltodextrin and soy protein isolate	- EE (%): > 93% - Particle size: : ~ 6,4 – 10,9 µm - % inhibition of degradation of microencapsulated anthocyanins was higher (43.8%) than non-encapsulated juice (35.2%)	(FREDES et al., 2018)
	Thai rice bran	Gelatin and gum arabic	- EE (%): 93,45 – 95,09 % - Particle size: 0,24 – 4,42 µm - Microparticles produced with gelatin showed a higher % release (83.77%) compared to the free extract (45.01%) and those produced with gum arabic (43.29%) during in vitro digestion.	(PEANPARKDEE et al., 2021)
Spray chiling	Grape skin anthocyanins	Palm oil (PO) and fully hydrogenated palm oil (FHPO)	- EE (%): 61,3 – 81,5 % - Particle size: 23,30 - 30,12 µm -The free extract released more than 70% of the anthocyanins, while the microencapsulated extract showed a controlled release. - The maximum release of anthocyanins from lipid microparticles ranged from 12 to 2%, depending on the proportion of encapsulating materials (FHPO and PO). - Lipids with a higher melting point (FHPO) tend to provide greater physical resistance to the microparticles, collaborating to control the release of anthocyanins.	(DE ABREU FIGUEIREDO et al., 2023)
Molecular Inclusion	Cyanidin-3-glucoside and anthocyanins from blackberry purees	β-cyclodextrin	- 37 and 44% of the total anthocyanins were retained in unfortified and β-CD fortified blackberry purees after in vitro digestion.	(FERNANDES et al., 2018)
	Anthocyanins from *Saffron Petals*	β-cyclodextrin	- EE (%): ~ 63% - Particle size: 32.04- 141.98 µm - Controlled release of anthocyanins in in vitro digestion - After 4 hours of digestion 80.33 ± 1.52% of the anthocyanins were released	(AHMAD et al., 2018)
	Anthocyanins from *Berberis lycium*	β-cyclodextrin	-The bioaccessibility for free anthocyanins and inclusion complexes were similar in the gastric condition (17.5 and 18.4%) and in the intestinal condition (9.2 and 9.7%)	(PRADHAN et al., 2022)

Liposomes	Acai anthocyanins	Soy lecithin and terpenes	- Increased uptake rate in Caco-2 cells - The transport of cyanidin-3-glucoside and cyanidin-3-rutinoside anthocyanins increased by 206% and 235% with a concentration of 5000 mg/L of soy lecithin	(CARDONA; MERTENS-TALCOTT; TALCOTT, 2015)
	Blackberry anthocyanins	Soy lecithin and chitosan	- Particle size: 0,3 - 80 µm - Trapping of anthocyanins in liposomes increased their recovery in samples (2.1%) at the end of digestion compared to control anthocyanins (1.8%) - The % recovery of anthocyanins from the chitosan-coated liposome was the highest (3.7%)	(GÜLTEKIN-ÖZGÜVEN et al., 2016)
	Blueberry anthocyanins	Soy lecithin and cholesterol	- EE (%): ~ 50% - Particle size: ~ 159 nm - Low protection to anthocyanins in the intestinal digestion phase - More than 90% of anthocyanins were released into the intestinal environment after 4 h	(ZHAO; TEMELLI; CHEN, 2017)
	Cyanidin-3-glucoside (C3G)	Phosphatidylcholine and cholesterol	- EE (%): ~ 70 % - Particle size: ~166 nm - 18.6% of C3G was released from nanoliposomes in 4 gastric digestion - Approximately 35.6% of C3G was released from nanoliposomes within 4 h in intestinal digestion	(LIANG et al., 2017)
	Purple sweet potato anthocyanins	Hydrogenated soy phosphatidylcholine	- Greater recovery of encapsulated anthocyanins at the end of 12 h of digestion (6.43 ± 0.32 %) compared to free anthocyanins (2.35 ± 0.12 %) - Increased absorption rate in Caco-2 cells	(CHENG et al., 2018)
	Cyanidin-3-glucoside and peonidin-3-glucoside	Soy lecithin, cholesterol and tween 80	- EE (%): ~ 91 % - Particle size: ~53 nm - Anthocyanins from nanoliposomes exhibit sustained release and high stability during in vitro digestion - Retention rates: 72.76% for encapsulated anthocyanins and 52.01% for free ones	(CHI et al., 2019)
	Cyanidin-3-glucoside and peonidin-3-glucoside	Lecitina de soja, colesterol e tween 80	- EE (%): ~ 92 % - Particle size: ~54 nm - Improved stability of anthocyanins in the alkaline environment of the cell medium - Greater absorption of anthocyanins in liposomes (0.42 ± 0.02 µg/mg of protein) than non-encapsulated anthocyanins (0.22 ± 0.03 µg/mg of protein)	(SUN et al., 2021)

Niosomes	Blueberry anthocyanins	Soy lecithin and cholesterol	- EE (%): ~ 82 % - Particle size: ~211 nm - After 2.5 h, about 54.85% of the anthocyanins were released from the liposome during in vitro intestinal digestion	(WANG et al., 2021a)
	Anthocyanin-rich black carrot extract	Cholesterol and tween 20	- EE (%): ~ 40 % - Particle size: ~130 – 330 nm - Approximately 90% of the anthocyanins were released at the end of 10 hours and the rest at the end of 5 days.	(FIDAN-YARDIMCI et al., 2019)
Ionic Gelation	Hibiscus extract	Pectin, rapeseed oil and $CaCl_2$	- The release of anthocyanins from the particles into the gastric fluid reached a maximum of 13.5%, - In the intestinal fluid, after 20 min, the particles disintegrated releasing anthocyanins	(MOURA et al., 2019)
	Grape peel extract	Sodium alginate, $CaCO_3$ and soybean oil	- EE (%): ~ 70-75 % - Particle size: ~0,5 – 99 µm - At the end of gastric digestion, retention of anthocyanins for spray-dried powders (P-SD) and freeze-dried powders (P-FD was 83% and 70%, and for free anthocyanins 45% - In the last phase of intestinal digestion, the retention of anthocyanins was 1%, 15% and 24.5% for free anthocyanins, P-FD and P-SD	(ZHANG et al., 2020a)
	Anthocyanins from *Aronia melanocarpa*	Chitosan and sodium tripolyphosphate (TPP)	- EE (%): ~ 65 % - Particle size: 197 nm - The retention rate of free anthocyanins quickly decreased to 38.52%, while that of nanoparticles was 79.13% after simulated intestinal digestion.	(WANG et al., 2021b)
	Black carrot anthocyanins	Chitosan and sodium tripolyphosphate (TPP)	- EE (%): ~ 70 % - Particle size: 274 nm - Better antioxidant activity in vivo, indicating greater stability and bioavailability	(CHATTERJEE et al., 2021)
	Grape anthocyanin-rich extract	Alginate or pectin, $CaCl_2$ and soybean oil	- EE (%): 46-89 % - Particle size: ~11-22 µm - The combined strategy of ionic gelation with double emulsion improved the retention of anthocyanins in the gastric phase (less than 20% of anthocyanins released) and in the intestinal phase (around 40% of anthocyanins released) after 240 min	(NORCINO et al., 2022)
Coacervation	Blueberry	Chitosan	- EE (%): ~63 %	(HE et al., 2017)

anthocyanins	hydrochloride and carboxymethyl chitosan	- Particle size: ~219 nm - In intestinal digestion, the percentage of anthocyanins released from nanoparticles was 30.61%, compared to 50.49% of free anthocyanins	
Blackberry anthocyanins	Chitosan and alginate	- Particle size: ~500 µm - Between 50 and 90% of the anthocyanins were released into the gastric fluid at the end of 24 hours, while only around 20% of the anthocyanins were released into the intestinal fluid at the same time.	(KANOKPANONT; YAMDECH; ARAMWIT, 2018)
Blend of anthocyanins with a purity of 25%	Cloridrato de quitosana, carboximetilquitosana e β-Lactoglobulina	- EE (%): ~69 % - Particle size: ~91 nm - Less anthocyanin release than free anthocyanin solution - The residual anthocyanin concentration of chitosan/β-Lg nanocomplexes after 6 h of intestinal digestion incubation was 42.5 µg/mL and of nanoparticles without β-Lg of 28.5 µg/mL	(GE et al., 2019a)
Blueberry anthocyanins	Chitosan and pectin	- EE (%): ~67 % - Particle size: 100-300 nm - After 12 h of digestion, the rate of anthocyanin release from the nanocomplexes in gastric juice was 26%, while in intestinal juice it was 56%	(Zhao et al., 2020)
Anthocyanins from *Carissa carandas*	Chitosan and pectin	- EE (%): ~23-47 % - The bioaccessibility (%) of the coacervate formulation (24.3 ± 0.5) was significantly higher than the crude extract (12.2 ± 1.0) and purified anthocyanins (18.9 ± 0.7)	(SARKAR et al., 2021)
Cranberry anthocyanin extract	Chitosan hydrochloride, carboxymethyl chitosan and whey protein isolate	- EE (%): ~60 % - Particle size: ~332 nm - At the end of digestion, the concentration of non-encapsulated anthocyanins reduced from 233.67 µg/mL to only 0.26 µg/mL - For encapsulated anthocyanins, the concentration reduced from 230.66 µg/mL to 5.72 µg/mL.	(WANG et al., 2021c)
Blueberry extract	Chitosan and pectin	- EE (%): ~91 % - Particle size: ~81 nm - The rate of anthocyanin release from the particles was only 34.95% in gastric digestion - The encapsulated anthocyanins were almost completely released (99.10 ± 0.14%) at the end of 6 h intestinal digestion	(XIE et al., 2023)

3.3.1 Spray-drying

In spray drying, anthocyanins and an aqueous mixture of the wall material are converted into small droplets (ranging from 5 to 30 μm) that come into direct contact with a stream of hot, dry air (>100 °C). Upon contact with the air, the droplets lose water in the form of steam, resulting in the formation of dust particles (microcapsules).

Recent studies have reported that the bioavailability and bioaccessibility of anthocyanins can be improved by using the spray-drying microencapsulation technique (RIGHI DA ROSA et al., 2021; ROSALES-CHIMAL et al., 2023). Most of these studies utilize in vitro simulated digestion to evaluate the potential controlled release and subsequent increase in bioaccessibility. However, there are limited studies available that evaluate the bioavailability of these compounds in vivo.

The microcapsules produced in spray dryers are capable of delaying the release of anthocyanins and their potential degradation during the digestion process (Table 1). However, the bioaccessibility and bioavailability can vary significantly depending on the wall material used in the process, as these factors influence the solubility of the powder, the morphological structure of the capsule, and the chemical interaction with anthocyanins (DUMITRAŞCU et al., 2021; PAN et al., 2022). Flores et al. (2014) observed that during in vitro digestion, gum arabic microcapsules showed significantly higher anthocyanin release rates than whey protein microcapsules. In addition, gum arabic microcapsules had a higher anthocyanin content than whey proteins.

Different carboxymethyl starch (CMS)/xanthan gum (XG) ratios were evaluated and it was observed that the greater the amount of xanthan gum in the mixture, the lower the release of blueberry anthocyanins during simulated digestion (CAI et al., 2019). The release reached 21.24% for CMS/GX mixture (60/1 m/m) and 35.68% for CMS-only microcapsules. The authors attribute this fact to the formation of hydrogen bonds between CMS and XG, which enhances the interaction of the chains and protects the microcapsules against gastric acid. In the intestine, the release was greater than 70% in all samples and the percentage of release also decreased with the increase in the proportion of GX. When the pH increases, the carboxymethyl group of CMS can dissociate causing electrostatic repulsion that leads to microcapsule expansion and anthocyanin release (CAI et al., 2019).

Righi da Rosa et al. (2021) observed that a mixture of maltodextrin DE20 (5%), hi-maize (5%), inulin (5%), and gum arabic (5%) promoted an improvement in the delivery of anthocyanins in simulated gastrointestinal conditions, increasing their bioavailability compared to unencapsulated anthocyanin extract. Encapsulated anthocyanins showed less degradation compared to anthocyanin extract during in vitro gastric and intestinal digestions

In addition to carbohydrates, proteins are widely used in spray-drying encapsulation processes, since they can function as emulsifiers, gelling agents, and foamers in food systems. Pan et al. (2022) demonstrated that after in vitro gastric digestion, the percentage of the release of non-microencapsulated anthocyanins (88.5%) was significantly higher than microencapsulated anthocyanins with soy protein isolate (22.1%). Furthermore, shortly after in vitro intestinal digestion, 100% of the non-microencapsulated anthocyanins were released while the microcapsules released approximately 45%. Similarly, Liao et al., (2021), observed rapid degradation of non-microencapsulated anthocyanins during in vitro digestion. In addition, they also demonstrated that casein microcapsules exhibited a greater ability to extend the anthocyanin release process compared to whey protein isolate microcapsules.

A recent study evaluated the influence of blueberry extract encapsulation with whey protein on the intestinal bioavailability and bioaccessibility of anthocyanins in humans. Unlike the results presented so far, encapsulation did not have a positive effect on the stabilization of anthocyanins during intestinal passage. Furthermore, an increase in the rate of degradation of encapsulated anthocyanins was observed when compared to non-encapsulated materials. The detection of anthocyanins in the plasma of the volunteers was 28% lower for the encapsulated samples compared to the non-encapsulated extract. There was also a greater elimination of anthocyanins and their degradation products via urine than that observed after ingestion of pure blueberry extract (MUELLER et al., 2018).

Studies have shown that microencapsulation can effectively delay the release of anthocyanins and protect them from degradation during the digestion process. The choice of wall material plays a crucial role in determining the solubility, morphological structure, and chemical interaction with anthocyanins, thus influencing the bioaccessibility and bioavailability of the encapsulated compounds. Different combinations of wall materials, such as CMS/XG and maltodextrin, he-maize, inulin,

and gum arabic, showed potential to enhance the delivery and stability of anthocyanins during simulated gastrointestinal conditions. Future research should focus on assessing the bioavailability of encapsulated anthocyanins in vivo, providing a more comprehensive understanding of their potential health benefits and practical applications.

Table 2 - Positive and negative points of anthocyanin encapsulation techniques

Techniques	**Positive points**	**Negative points**
Spray-drying	High drying efficiency Control of process conditions Large scale application Possibility to apply different wall materials Increased bioaccessibility of anthocyanins	Loss of volatile compounds Possible thermal degradation Few in vivo studies/experiments
Spray chilling	Utilization of moderate temperatures Restriction of wall materials Increased bioaccessibility of anthocyanins	High cost No bioavailability studies
Lyophilization	Use of low temperatures / Ideal for thermolabile compounds	High cost
Molecular Inclusion	Controlled release Increased bioaccessibility of anthocyanins	Limited group of wall material Few studies
Liposomes	Controlled release	Difficulty in large-scale production Low long-term stability Heterogeneous particle size Few studies
Niossomes	Greater stability compared to liposomes Possibility of higher production	Possibility of higher production Few studies
Ionic gelation	Low cost Use of low temperatures / Ideal for thermolabile compounds Controlled release Extensive potential for material combination	Low long-term stability Heterogeneous particle size Few studies
Coacervation	Increased bioaccessibility of anthocyanins High encapsulation efficiency Use of low temperatures / Ideal for thermolabile compounds	Low long-term stability Heterogeneous particle size No bioavailability studies

3.3.2. Spray chilling

In spray chilling, anthocyanins are mixed with a lipid matrix, which can be a molten lipid (more common) or a water-in-oil emulsion (BERTONI et al., 2019). Then, this dispersion is atomized with the aid of a heated spray nozzle, forming drops. These drops come into contact with a cooling medium (cold air or liquid nitrogen, ~1 °C) and solidify, forming solid lipid microcapsules (FIGUEIREDO et al., 2022). The wall materials used in this technique are waxes, triacylglycerols, fatty acids, or mixtures thereof, with melting points ranging from 36 to 75°C (ORIANI et al., 2016). One of the main advantages of this method is that the microparticles are produced at mild temperatures and can be used to encapsulate thermolabile compounds. Additionally, they have high added value and can be employed in food matrices with antioxidant, coloring, and flavoring functions, enabling the controlled release of these compounds (NAHUM & DOMB, 2021).

Microencapsulation by spray chilling was effectively employed to enhance the bioaccessibility of grape skin anthocyanins. The authors observed that palm oil (PO) combined with fully hydrogenated palm oil (FHPO) promoted a protective effect on anthocyanins, contributing to a controlled release of this compound (DE ABREU FIGUEIREDO et al., 2023). In the simulation test of the gastric environment of the stomach, it was observed that within the first 20 minutes, approximately 70% of the anthocyanins from the free extract were already released. On the other hand, the release of microencapsulated anthocyanins ranged from 5 to 9% during the same period. In the intestinal simulation, both the free extract and the microparticles reached maximum release values within the first 20 minutes, which may be related to the degradation of the active compound due to the pH change from 1.2 (stomach) to 7.4 (duodenum). However, for the free extract, a linear decrease was observed over time, unlike the microencapsulated anthocyanins, which exhibited controlled release. This release control was associated with the presence of lipid wall materials, which possibly provided greater physical resistance to the microparticles (DE ABREU FIGUEIREDO et al., 2023).

No studies were found evaluating the bioavailability of anthocyanins encapsulated using this technique. Additionally, so far, only one study has been identified for assessing the bioaccessibility of anthocyanins. Despite being considered a promising technique due to its ease of application on an industrial

scale, as the equipment and operating mechanism are closely related to spray drying, microencapsulation by spray chilling is still underutilized in the food industry. Although the temperature used for microcapsule formation is low, anthocyanins must be stable at the melting temperature of the wall material. Finding a lipid wall material with ideal properties for this process, such as a stable melting point for anthocyanins, oxidative stability, and food-grade characteristics, among others, is likely one of the biggest challenges in applying this technique for anthocyanin microencapsulation.

3.3.3. Lyophilization

Lyophilization is a drying process in which an aqueous mixture of anthocyanins and the wall material is frozen, and the ice is converted into vapor through sublimation (FANG & BHANDARI, 2012; GARCIA-AMEZQUITA et al., 2016). The final characteristics of the capsules, such as size, charge, and stability, are influenced by the formulation (anthocyanins and wall material) prepared before freezing (MUHOZA et al., 2023).

Microencapsulation of blueberry anthocyanins using a combination of carboxymethyl starch (CMS) and xanthan gum (XG) followed by lyophilization improved thermal stability and delayed degradation (CAI et al., 2019). In this study, approximately 30% and 70% of the anthocyanins present in the microcapsules were released during gastric and intestinal digestion, respectively, as simulated in an in vitro assay.

Another study evaluated the bioaccessibility of microencapsulated Maqui juice anthocyanins using lyophilization and spray-drying techniques, with maltodextrin and soy protein isolate as wall materials (FREDES et al., 2018). Bioaccessibility was calculated based on the percentage of inhibition of anthocyanin degradation during in vitro digestion simulation. Microencapsulated anthocyanins exhibited a higher percentage of inhibition (43.8% for lyophilized) compared to free juice (35.2%), indicating protection of the active compound and controlled release. The physical differences between the particles (sprayed or lyophilized) had no significant effect on stability. The authors attributed the low percentage of inhibition to the high degradation of anthocyanins under gastrointestinal conditions (pH change from 2 to 6).

The encapsulation of thai rice bran extract in gelatin or gum arabic with modified surface charge was also evaluated (PEANPARKDEE et al., 2021). After in vitro digestion, gelatin (positively charged) showed a high capacity to protect negatively charged compounds in rice bran extracts (phenolic acids, flavonoids, and anthocyanins). Lyophilized microparticles with gelatin resulted in a high release of compounds during gastrointestinal digestion (83.77%). Gum arabic (negatively charged) exhibited reduced release due to its surface charge (43.29%), even lower than that of the non-encapsulated extract (45.01%) (PEANPARKDEE et al., 2021).

Lyophilization is considered a suitable technique for anthocyanin encapsulation mainly because it utilizes low temperatures. However, the process can be costly as it requires specific equipment, a temperature-controlled environment, and technical expertise in setting appropriate parameters (e.g., temperature and pressure). Additionally, the process can be time-consuming, taking days to complete the dehydration of the material depending on the sample. Therefore, this technique is more suitable for applications where anthocyanins are used in high-value products.

3.3.4. Molecular Inclusion

The molecular inclusion technique is an encapsulation method in which anthocyanin or part of the molecule fits into a cavity-bearing host molecule through van der Waals forces, hydrogen bonds or hydrophobic interactions (JOYE; MCCLEMENTS, 2014). Cyclodextrins (CDs) are the most widely used materials to form inclusion complexes with other substances, with β-cyclodextrins being the most used form in the food sector due to their availability, safety and cost (MOHAMMADALINEJHAD; KUREK, 2021). There are different methods to prepare inclusion complexes, such as co-precipitation, supercritical carbon dioxide, grinding, microwave irradiation and spray-dryer (CID-SAMAMED et al., 2022).

Due to its structural characteristics and potential to form inclusion complexes, β-cyclodextrin has been studied as a candidate to encapsulate anthocyanins and reduce their losses, preserving them from polymerization, oxidation and degradation reactions, being a potential way to increase concentrations of bioactive anthocyanins in the gastric tract (Table 1) (FERNANDES et al., 2018; MOHAMMADALINEJHAD; KUREK, 2021). However, the chemical form of anthocyanins has a significant influence on the efficiency of inclusion complex formation with cyclodextrins. Anthocyanins in the flavilium cation form are not entirely encapsulated in the

cyclodextrin cavity because of their planar structure and polarity (MOHAMMADALINEJHAD; KUREK, 2021). In contrast, more flexible colorless and neutral forms are more favorable to forming inclusion complexes (HOWARD et al., 2013; MOHAMMADALINEJHAD; KUREK, 2021). An anti-copigmentation effect (discoloration of anthocyanins) has also been reported as a result of the complexation of β-CD with anthocyanins, which is also indicative of the preferential interaction between β-CD and anthocyanins in the hemiketal form (FERNANDES et al., 2018).

The molecular inclusion technique with β-cyclodextrin (β-CD) was used to evaluate the stability of cyanidin-3-glucoside and anthocyanins in blackberry purees (FERNANDES et al., 2018). In this study, it was demonstrated that complexation with β-CD delayed the degradation of anthocyanins in blackberry purees during in vitro digestion. In the gastric phase, the stability of anthocyanins in purees fortified with β-CD was like unfortified purees. After intestinal digestion, approximately 37 and 44% of the total anthocyanins were retained in unfortified and fortified blackberry purees, respectively. The authors indicated an increase in the bioaccessibility of anthocyanins with the addition of β-CD (FERNANDES et al., 2018).

Encapsulation of anthocyanins from saffron petals with β-CD prevented the release of these compounds in gastric conditions and resulted in greater control of anthocyanin release in intestinal digestion (AHMAD et al., 2018). In contrast, an inclusion complex of anthocyanins from Berberis lycium with β-cyclodextrin (β-CD) demonstrated successful inclusion. However, it had no significant effect on the bioaccessibility of anthocyanins (PRADHAN et al., 2022).

Regarding the impact of anthocyanin encapsulation with β-CD on colonic fermentation, the results of a study with cyanidin-3-glucoside, delphinidin-3-glucoside and malvidin-3-glucoside showed that β-CD ensures constant release and sustained release of anthocyanins in the colon, which may improve their in vivo bioavailability. Furthermore, a bacterial interaction with the released anthocyanins was observed, suggesting that anthocyanins and their metabolites may positively modulate the intestinal bacterial population (FLORES et al., 2015).

3.3.5. Lipossomes

Liposomes are spherical-shaped microscopic structures with one or more phospholipid bilayer membranes (DEB et al., 2019). Liposomes are often employed in compound delivery systems and can incorporate hydrophobic and hydrophilic compounds. Hydrophilic molecules are incorporated into the inner core of liposomes, while hydrophobic molecules tend to remain in the lipid portion of the phospholipid bilayer (SINGH; ZEALAND, 2012).

The application of liposomes in the food area has been considered one of the main strategies to promote the controlled release, improve the stability of bioactive compounds under different conditions and increase the absorption of compounds (LIU et al., 2020). Due to their amphipathic nature, phospholipids are soluble in both oily and aqueous media and can help in the absorption and increase the bioavailability of compounds such as anthocyanins, which are highly polar and difficult to cross the lipid outer membrane of enterocytes from the small intestine (CHENG et al., 2018).

As evidenced, incorporating anthocyanins in liposomes increases their stability during digestion and intestinal absorption rate in cellular models (Table 1). For purple sweet potato anthocyanins, complexation with phospholipids (hydrogenated soybean phosphatidylcholine) resulted in significantly higher anthocyanin recovery at the end of in vitro digestion, with approximate values of 41.7% and 6.4% after 3 and 12 hours of digestion (CHENG et al., 2018). Furthermore, apparent permeability coefficient (Papp) values in Caco-2 cells were significantly higher for anthocyanins complexed with phospholipids than for free anthocyanins (CHENG et al., 2018).

Similar results were observed for nanoliposomes formed with an extract rich in cyanidin-3-glucoside and peonidin-3-glucoside, soy lecithin, cholesterol and Tween 80 (SUN et al., 2021). The retention rate of encapsulated anthocyanins was 88.2%, while the retention was 73.2% for free anthocyanins. The absorption of anthocyanins in liposomes was also higher (0.42 μg/mg protein) than non-encapsulated anthocyanins (0.22 μg/mg protein) (SUN et al., 2021).

For açai anthocyanins encapsulated with phospholipids and terpenes, the transport of cyanidin-3-glucoside (1.38%) was slightly higher than that of cyanidin-3-rutinoside (1.06%) (CARDONA; MERTENS-TALCOTT; TALCOTT, 2015). The overall transport of anthocyanins encapsulated with soy lecithin (500 mg/L) was not

significantly increased compared to unencapsulated samples. However, when the concentration was increased to 5000 mg/L, the transport of cyanidin-3-glucoside and cyanidin-3-rutinoside increased by 206% and 235%, respectively. According to the authors, this increase can be attributed to the encapsulation of compounds due to the formation of aggregates, which protect anthocyanins from degradation and allow their transport through the monolayer (CARDONA; MERTENS-TALCOTT; TALCOTT, 2015).

In another study, with liposomal encapsulation of blueberry anthocyanins with soy lecithin and cholesterol, more than 90% of anthocyanins were released into the intestinal medium after 4 h (ZHAO; TEMELLI; CHEN, 2017). The low protection observed was related to the rapid enzymatic hydrolysis of phospholipids by pancreatin and the rapid degradation of anthocyanins released from the liposome in the middle of the small intestine. Liposomes are disrupted by bile salts that function as surfactants during digestion in the gastrointestinal tract (GÜLTEKIN-ÖZGÜVEN et al., 2016).

The instability of liposomes can lead to reduced effectiveness in encapsulation, with oxidation and hydrolysis being the two main mechanisms associated with the degradation of these structures (NAKHAEI et al., 2021). Furthermore, liposomes can be strongly affected by physicochemical characteristics, including bilayer membrane composition, size, stiffness, and charge (NAKHAEI et al., 2021). Another factor that affects stability is lipid composition, with the retention of compounds by liposomes affected by the fluidity of the liposomal membrane and composition (NAKHAEI et al., 2021). The proportion of anthocyanins used in the formation of liposomes also affects the stability of the system. Thus an adequate proportion of phospholipid for the bioactive tends to generate a more stable structure (SHEN et al., 2022).

In general, phospholipids can protect anthocyanins from degradation, resulting in greater bioaccessibility at the end of digestion and increased bioavailability. However, the stability of liposomes can be affected by factors such as lipid composition, bilayer membrane characteristics and enzymatic hydrolysis. More research is needed to explore the potential of this technique considering its influence on the controlled release, stability and bioavailability of anthocyanins.

3.3.6. Niosomes

Niosomes are lipid-based structures employed in the delivery of bioactive compounds composed mainly of hydrated nonionic surfactants, cholesterol or its derivatives (KAUR, 2016). The advantages of niosomes, compared to liposomes, are greater stability, low cost, ease of formulation and scale-up (GE et al., 2019b; KARIM et al., 2010). Due to their lipid nature, niosomes can also help increase the absorption of anthocyanins since surfactants can increase the permeability of the intestinal membrane in a concentration-dependent manner (FIDAN-YARDIMCI et al., 2019).

Niosomal formulations with cholesterol and tween 20 to encapsulate anthocyanin-rich black carrot extract was developed and evaluated for in vitro release profile (FIDAN-YARDIMCI et al., 2019). The anthocyanin release profile of the niosomal formulation was investigated for 7 days. According to the authors, approximately 90% of the anthocyanins were released from the niosomes at the end of 10 h and the rest at the end of 5 days (FIDAN-YARDIMCI et al., 2019).

In this review, only one study evaluating the encapsulation of anthocyanins in niosomes was found. Despite being considered a promising technique, it still requires further studies to overcome its limitations. Niosomes have higher chemical stability than liposomes, however, both liposomes and niosomes have physical stability problems. Aggregation of vesicles, fusion of two layers and high permeability of compounds are the most common physical alterations (ABDELKADER; ALANI; ALANY, 2014; GHARBAVI et al., 2018). In addition, the surfactant compounds used in the production of niosomes may be toxic, which also limits their applicability (FIDAN-YARDIMCI et al., 2019; GHARBAVI et al., 2018).

3.3.7. Ionic gelation

Ionic gelation is a technique used to synthesize capsules based on the electrostatic interaction between a polymer and a polyelectrolyte (BAYRAKTAR et al., 2017). When the bioactive compound is added to the reaction, it can be trapped or adsorbed within the matrix structure formed from the polymer crosslinking (PEDROSO-SANTANA; FLEITAS-SALAZAR, 2020). Ionic gelation can be obtained through atomization, dripping (co-extrusion, extrusion), electrostatic spraying processes and jet-cutting (MOHAMMADALINEJHAD; KUREK, 2021). Variables such

as pH or ionic strength, agitation rate, flow rate and the temperature at which the process occurs directly influence the formation of capsules (BAYRAKTAR et al., 2017; SACCO et al., 2021).

The ionic gelation technique is considered low-cost, as it does not require the use of advanced equipment, high temperatures or organic solvents in the method. However, the use of this technique for the encapsulation of hydrophilic or low molecular weight compounds is considered a challenge due to the miscibility of wall materials with hydrophilic cores, which makes retention and controlled release of the active difficult (KUROZAWA; HUBINGER, 2017; MOHAMMADALINEJHAD; KUREK, 2021).

Chitosan, sodium alginate and pectin are among the most used polymers for encapsulating bioactive compounds, such as anthocyanins, through the ionic gelation technique. Chitosan is usually used in combination with sodium tripolyphosphate (TPP), as the positively charged chitosan amino groups cross-link in the presence of negatively charged TPP phosphate groups to form particles (SACCO et al., 2016). While calcium chloride (CaCl2) is one of the most frequently used agents for cross-linking with the anionic groups of alginate and pectin (LEE et al., 2012; SECCHI et al., 2014).

Studies with promising results indicate that encapsulation with chitosan can improve the stability of anthocyanins and prevent their degradation during passage through the gastrointestinal tract (Table 1). For anthocyanins from Aronia melanocarpa nano-encapsulated with chitosan and sodium tripolyphosphate, the retention rate of anthocyanins (96%) in the nanocapsules was clearly higher than free anthocyanins (88%) when the samples were submitted to gastric digestion for 1 h (WANG et al., 2021b). Furthermore, the retention rate of free anthocyanins rapidly decreased to approximately 39%, while that of nano-encapsulated was ~79% after simulated intestinal digestion (WANG et al., 2021b).

After entering the gastrointestinal tract, chitosan capsules are exposed to different pH values, ions and digestive enzymes, which can affect the effectiveness of the capsules in releasing the compounds (LI et al., 2015). A change in the external pH environment in which the capsules are dispersed can change the charge of the wall materials and affect the electrostatic attraction between them and the encapsulated bioactive. Although chitosan encapsulation may improve the stability of anthocyanins, this polymer can be dissociated at low pH values, such as that of the

stomach (pH = 2.0), leading to the release of anthocyanins and limiting their delivery and absorption in the intestinal mucosa (KIM et al., 2019). On the other hand, chitosan has low solubility in neutral environments, which helps to preserve the structure of nanocapsules under these conditions. The more stable the ionic bond between chitosan and the cross-linking agent in the capsules, the less the influence of pH. Thus, the degradation of nanocapsules can be delayed resulting in the sustained release of core materials (KIM et al., 2019; YAN et al., 2018).

When working with hydrophilic compounds, techniques such as double emulsion can be employed to improve encapsulation characteristics by ionic gelation (MOHAMMADALINEJHAD; KUREK, 2021). Blueberry anthocyanins were encapsulated using a double emulsion (aqueous pectin-rapeseed oil and calcium chloride solutions) (MOURA et al., 2019). The release of anthocyanins from the capsules into the gastric fluid reached a maximum of 13.5%, while for the free extract, 95% of the anthocyanins were detected in the medium in the first 15 minutes of the test. On the other hand, with 20 min in the intestinal phase, the produced capsules disintegrated, releasing the anthocyanins, which was observed by the color changes in the medium due to the pH of 7.4. During the test with free extract, it was observed that, after 10 min, the anthocyanins had already been degraded (MOURA et al., 2019).

In another study, an anthocyanin-rich extract of grapes was encapsulated with alginate and pectin by the ultrasonic ionic gelation technique, also combined with emulsification (NORCINO et al., 2022). Pectin and alginate had a low protective effect on anthocyanins during gastric digestion, as more than 45% of this pigment was released in the first 20 min. On the other hand, capsules prepared using double emulsion and ionic gelling showed less release in simulated gastric fluid (< 20%) than capsules prepared with a non-combined strategy. In the intestinal phase, capsules with pectin and alginate released more than 60% of anthocyanins at the final time of 240 min, while capsules combined with emulsion released less than 40% at the end of digestion (NORCINO et al., 2022).

The lower protection observed for anthocyanins encapsulated only by ionic gelation with alginate and pectin may be associated with the porosity of the formed gels, which allows the migration of bioactive compounds to the medium, mainly of small molecules, resulting in low protection. Meanwhile, the association with techniques such as the double emulsion decreases the release of anthocyanins in

the gastric phase, as the oil phase acts as a barrier and delays the release of bioactive compounds. However, in the intestinal phase, oil lipolysis occurs through the action of pancreatic lipases, which leads to the demulsification of microcapsules and the release of anthocyanins. Despite this, the combination of techniques was still more efficient in delaying the release of anthocyanins than the isolated ionic gelation technique. The differences in behavior observed for the different polymers can be attributed to the physical-chemical interactions that occur between the polymers and the anthocyanins and also with the oil phase, which leads to the formation of a more complex structure with less porosity (NORCINO et al., 2022).

Researches evaluating the impact of encapsulation by ionic gelation on the bioavailability of anthocyanins are scarce. In a study with chitosan-encapsulated black carrot anthocyanins, the in vivo antioxidant potential in rats from encapsulated and non-encapsulated samples was evaluated (CHATTERJEE et al., 2021). The results indicated that the in vivo antioxidant potential of anthocyanins was improved after nanoencapsulation, as observed in serum antioxidant assays. Thus, the nanoencapsulation process may have improved the stability of anthocyanins in the gastric tract and even provided greater bioavailability of these compounds (CHATTERJEE et al., 2021).

3.3.8. Coacervation

Coacervation is a process that involves the formation of two separate liquid phases, a polymer-rich phase, called coacervate, and a polymer-depleted phase, which is the equilibrium solution (TIMILSENA et al., 2019). Depending on the number of polymers involved and the phase separation mechanism, coacervation can be classified into two types: simple or complex coacervation. In simple coacervation, only one polymer is employed, and the coacervates are formed through a dehydration mechanism caused by the addition of a salt or desolvation liquid in the reaction medium (TIMILSENA et al., 2019). While complex coacervation occurs through electrostatic interactions between two or more polymeric solutions with opposite charges, resulting in the formation of two immiscible liquid phases (YAN; ZHANG, 2014).

The molecules normally used in coacervation are proteins and polysaccharides, so this technique has great potential for application in food products. The proteins

used can be of animal origin, such as gelatin, whey proteins and egg albumin, or vegetable origin, such as soy, pea, wheat, lentil and chia protein (TIMILSENA et al., 2019). Among the most used polysaccharides are alginate, chitosan, gum arabic, pectin, agar-agar, carrageenan and carboxymethylcellulose (TIMILSENA et al., 2019).

There are several studies involving the application of coacervation for the encapsulation of anthocyanins. However, no studies were found that evaluated the impact of this form of encapsulation on the bioavailability of anthocyanins, only on bioaccessibility (Table 1). The encapsulation of blueberry anthocyanins with chitosan and pectin by the complex coacervation method delayed the release of anthocyanins in the gastric environment, which indicated that the capsules could enter the intestine without the expressive release of the bioactive in the stomach (ZHAO et al., 2020). After 12 h of digestion, the anthocyanin release rate in gastric juice was 26%, while in intestinal juice, it was 56% (X. Zhao et al., 2020). In another study with coacervates prepared with different combinations of pectin and chitosan, the bioaccessibility of the coacervate formulation (~24%) was significantly higher than the crude extract (~12%), as well as that of purified anthocyanins (~19%) (SARKAR et al., 2021).

The stability of anthocyanins extracted from blackberry was evaluated after encapsulation with combinations of alginate and chitosan in two different processes: dripping a sodium alginate solution into a bath of CaCl2 and chitosan and dripping a sodium alginate solution into a bath of CaCl2 followed by incubation of the Ca-alginate capsules in a chitosan solution (KANOKPANONT; YAMDECH; ARAMWIT, 2018). In that study, the formed capsules degraded and released anthocyanins faster in the gastric fluid than in the intestinal phase. Between 50 and 90% of the anthocyanins were released in the gastric fluid at the end of 24 hours, while about 20% of the anthocyanins were released in the intestinal fluid at the same time interval. The more significant release of anthocyanins in the gastric phase was associated with the method used to form the capsules. In the incubation of calcium alginate in chitosan solution, a chitosan coating was formed around the formed capsules and, according to the authors, the possible electrostatic interaction between anthocyanins (positively charged) and alginate (negatively charged) helped in the retention of anthocyanins inside the capsules.

Carboxymethyl chitosan (negatively charged) and chitosan hydrochloride (positively charged) are two water-soluble chitosan derivatives that can be used for

encapsulation of anthocyanins via coacervation (LIANG et al., 2010; WANG et al., 2021c). Chitosan hydrochloride and carboxymethyl chitosan nanoparticles loaded with blueberry anthocyanins showed slower degradation during in vitro digestion (HE et al., 2017). The rate of anthocyanins released from chitosan nanoparticles (~48%) was lower than that of free anthocyanins (~69%) in solution during gastric digestion. On intestinal digestion, the percentage of anthocyanins released from the nanoparticles was ~31%, compared to ~50% of free anthocyanins. The results showed that chitosan nanoparticles loaded with anthocyanins could reduce the release rate and reduce the degradation of anthocyanins in the gastrointestinal tract (HE et al., 2017).

In another study of the encapsulation of anthocyanins with chitosan hydrochloride and carboxymethyl chitosan, the β-lactoglobulin protein was included as a wall material in an attempt to reduce the instability of chitosan in the pH of the stomach and favor the transport of bioactive for the small intestine (GE et al., 2019a). Compared to unencapsulated anthocyanins, nanoparticles delayed the release of anthocyanins, with the sustained release property of nanoparticles with β-lactoglobulin superior to nanoparticles with chitosan alone. In in vitro digestion assays, it was found that the concentration of anthocyanins in aqueous solution (non-encapsulated form) and nanoparticles did not exhibit a significant change after gastric digestion. The residual anthocyanin concentration of chitosan/ β-lactoglobulin nanoparticles after 6 h of intestinal digestion incubation was 42.5 μg/mL, while that of nanoparticles without β-lactoglobulin was 28.5 μg/mL (GE et al., 2019a).

The release of blackberry anthocyanins in simulated gastric fluid and simulated intestinal fluid was also compared between the unencapsulated form and the encapsulated form with 3 polymers: chitosan hydrochloride, carboxymethyl chitosan and whey protein isolate (WANG et al., 2021c). Unencapsulated anthocyanins showed a rapid release in gastric digestion, with a final release of 82% and in intestinal digestion of ~21%. The encapsulation with the 3 polymers delayed the release of anthocyanins in gastric (53.5%) and intestinal (9.4%) conditions. At the end of digestion, the concentration of non-encapsulated anthocyanins reduced from 233.67 μg/mL to only 0.26 μg/mL. In contrast, for encapsulated anthocyanins, the concentration reduced from 230.66 μg/mL to 5.72 μg/mL. Thus, the bilayer coating formed improved the stability of anthocyanins in the pH of the intestine (WANG et al., 2021c).

4. FINAL CONSIDERATIONS

Strategies to enhance the bioaccessibility and bioavailability of anthocyanins have been extensively researched. Among them, the effects of the matrix, co-ingestion of food, processing, and microencapsulation can be mentioned. These strategies can have either positive or negative impacts on bioaccessibility and bioavailability, depending on the specific studies that highlight the best approaches to ensure greater benefits for individuals consuming foods rich in anthocyanins.

Numerous studies have primarily demonstrated an increase in the in vitro bioaccessibility of anthocyanins during digestion, with particularly promising results observed for microencapsulation. However, data regarding the impact on the bioavailability of anthocyanins are scarce for almost all techniques. Most studies have been conducted using experimental in vitro digestion systems, focusing on the degradation behavior and controlled release of anthocyanins in the gastric and intestinal phases. Only a few studies have utilized cellular, animal, and human models to evaluate whether the employed strategies indeed have an effect on the bioavailability of anthocyanins.

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Paper 2

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INFLUENCE OF SPONTANEOUS AND INOCULATED FERMENTATION OF AÇAI ON SIMULATED DIGESTION, ANTIOXIDANT CAPACITY AND CYTOTOXIC ACTIVITY

Abstract

This work describes the kinetic study of different types (spontaneous, lactic and alcoholic) of açai fermentation in terms of total phenolics and total anthocyanins, as well as antioxidant capacity, before and after simulated digestion (SD). Cytotoxicity (A549, HCT8 and IMR90 cells) and formation of reactive oxygen species (A549 cells) were also evaluated. The results revealed that spontaneous fermentation (SF) for 24 h, followed by SD, generated a product with greater bioaccessibility of phenolics (52.68%) and cyanidin-3 glycoside (27.01%) than unfermented açai. Likewise, lactic fermentation (LF) for 72 h improved the bioavailability of phenolics (64.49%) and cyanidin -3 - rutinoside (20.00%). On the other hand, alcoholic fermentation (AF) decreased the bioaccessibility of phenolic compounds and anthocyanins after SD. The SF 24h (10.16±1.25 μmol Trolox /g) and LF 72 h (15.90±0.51 μmol Trolox /g) significantly increased the antioxidant capacity after SD, when compared to unfermented açai (SF 0h, 4.00±0.09 μmol Trolox /g; LF 0h, 10.57±0.91μmol Trolox /g). It was concluded that the samples did not show cytotoxicity in the cell lines tested and, in addition, AF 24 h showed antioxidant and antimutagenic effects in vitro, reducing about 40% of chromosomal aberrations. The results obtained provide important information that can be used to produce foods with greater bioaccessibility of bioactive compounds.

Keywords: Açai fermentation; Bioaccessibility; Simulated digestion in vitro; Lactic acid bacteria; yeast; Cytotoxicity; ROS generation.

1. INTRODUCTION

Açai (Euterpe oleracea Mart.) is a fruit from the Brazilian Amazon, with a unique flavor appreciated in Brazil and worldwide. Its consumption increased over the last ten years mainly due to the health benefits associated with its chemical composition, especially the presence of bioactive substances, such as phenolics, flavonoids, and anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) (FREITAS et al., 2015; MACHADO et al., 2019).

Anthocyanins are natural pigments boasting high antioxidant capacity; therefore, they can figureht free radicals generated by human metabolism, thus preventing the development of various diseases. Recent studies have shown that the consumption of anthocyanin-rich foods has benefits against cancer (FRAGOSO et al., 2018; MANNINO et al., 2020; PIRARUCU et al., 2017), diabetes (ALNAJJAR et al., 2019; QUATRIN et al., 2018), obesity (ARANHA et al., 2019; THOMPSON et al., 2017; VUGIC et al., 2020) and neurocognitive diseases (KRIKORIAN et al., 2011, 2019).

However, the beneficial effects associated with the consumption of phenolic compounds, including anthocyanins, may be affected by their low bioavailability (DA SILVA HAAS et al., 2019; FARIA et al., 2013b). Several factors can affect the absorption of anthocyanins, such as their own structure, mainly the complexity of the glycoside linked to it (OLIVEIRA et al., 2019b); the food matrix, including the presence of sugar and protein; and the presence of other bioactive substances, which can compete for the same absorption site (CEBECI; ŞAHIN-YESILCUBUK, 2014; MULLEN et al., 2008; PHAN; BUCKNALL; ARCOT, 2019b; RIBNICKY et al., 2014; SENGUL; SUREK; NILUFER-ERDIL, 2014).

Therefore, investigating ways to increase the bioaccessibility and bioavailability of these compounds is necessary to increase the functional potential of these foods through processing. There is evidence that the fermentation process changes the anthocyanin profile and increases the content of total phenolic and the antioxidant capacity of food (BRAGA et al., 2018a; GAO et al., 2022; JUAN; WU; CHOU, 2010). This is mainly associated to the activity of microbial enzymes, which convert anthocyanins into other bioactive compounds such as lower-molecular weight phenolic acids. These compounds may have greater bioavailability and different biological activities (Braga et al. 2018; Guergoletto et al. 2016; Yan et al.

2019; Wiczkowski, Szawara-Nowak, and Topolska 2015). Although there are studies on the effect of fermentation on the profile and bioavailability of phenolic compounds, there are still no reports about the effect of fermentation on açai anthocyanins.

The main objective of this work was to evaluate the effect of three types of fermentation (spontaneous, lactic, and alcoholic) on the in vitro bioaccessibility of anthocyanins and antioxidant activity of those products. In addition, three cell lines were used to evaluate the cytotoxic effect of fermented açai extracts before and after simulated gastrointestinal digestion. Our work also contributes to the standardization of the açai's fermentation process, since the spontaneously fermented açai is a traditional food consumed locally in açai-producing regions.

2. MATERIALS AND METHODS

2.1. Chemical and cell cultures

ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), DPPH (1,1,-diphenyl-2-picrylhydrazyl radical), Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), Folin-Ciocalteu reagent, pepsin, pancreatin, and bile extract were obtained from Sigma-Aldrich Chemical, S.A. Dichloro-dihydro-fluorescein diacetate (DCFH-DA), 3-4,5 dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT), and Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM) were purchased from Sigma-Aldrich (São Paulo, Brazil). The other chemicals were of analytical grade. Aqueous solutions were prepared using ultrapure water (Millipore, São Paulo, Brazil).

The lung adenocarcinoma epithelial cells (A549), human ileocecal adenocarcinoma cells (HCT8) and normal human lung fibroblast (IMR90) were obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil). Cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM), supplemented with heat-inactivated fetal bovine serum (Gibco, EUA) to a final concentration of 10% (w/v) (HCT8, A549) and 20% (w/v) (IMR90). All these cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Açai fermentation

Açai was collected in the city of Ilha das Onças (Pará, Brazil). Immediately after collection, the fruits were pulped, frozen at -10 ºC and sent in a thermal box to the laboratory, where it was stored at -80 ºC until analysis.

The açai pulp was submitted to three different fermentations: spontaneous (SF); inoculated with the lactic bacterium Lactobacillus acidophilus for lactic fermentation (LF); or inoculated with the yeast Saccharomyces cerevisiae for induction of alcoholic fermentation (AF).

For SF, 2 kg of açai pulp were thawed and disposed into a closed container with gas outlet at 25 °C for 72 hours. For LF and AF, the respective inoculum was added at a concentration of approximately 106 CFU /mL (LF) or 106 cells /mL (AF). After homogenization, all mixtures were kept in closed containers at 25 °C for 72 h. Aliquots of 250 mL were collected at 0, 24, 48 and 72 h of fermentation (0–3 days) and analyzed for total phenolics, cyanidin-3-glucoside, cyanidin-3-rutinoside and antioxidant capacity.

The total acidity of the samples was determined by titration with standardized 0.01 N NaOH solution and phenolphthalein as indicator. The pH was determined by a previously calibrated pH meter (DIGIMED model Dm20).

2.3. Microbiological profile

Microbiological characterization was performed only for spontaneous fermentation. The aliquots were serially diluted for the measurement of lactic acid, acetic and mesophilic bacteria, and yeasts. For lactic acid bacteria count, Man Rogosa Sharpe Agar (Merck) was prepared with bromocresol blue (0.004% w/v), being considered as lactic bacteria the yellow (acid producing), catalase negative, and Gram-positive colonies. Acetic bacteria were counted using GYC agar (glucose 50 g /L, yeast extract 10 g /L, calcium carbonate 5 g /L, and agar 20 g /L) with ethanol (70 g /L). Finally, Potato Dextrose Agar (PDA - Merck) was used for the yeast count and Plate Count Agar (PCA - Merck) for mesophilic bacteria. The plates were incubated at 30 °C for 48 h under aerobic conditions, except for lactic bacteria, whose plates were incubated in microaerophilic cells. The results were expressed as CFU /mL.

1.1. Extraction of phenolic compounds

The phenolic extracts were obtained through the dilution of 50 mL of each sample with 500 mL of 70% ethanol solution (v/v) and stored under refrigeration (5 ± 1 °C) for 24 h. The mixture was vacuum-filtered using Whatman filter nº. 1 paper.

After this step, evaporation was performed to eliminate the alcohol. The obtained concentrates were quantitatively transferred to a volumetric flask and completed with water. The obtained extracts were stored at -80 °C until analysis.

2.4. Total phenolic determination

The quantification of total phenolic in the extracts derived from each aliquot of açai fermentation was performed by the colorimetric Folin-Ciocalteu assay (SINGLETON; ROSSI, 1965). A UV–Visible spectrophotometer (Model UV-M519) was used to measure absorbance at 760 nm. Gallic acid was used to build analytical curves, and total phenolic contents were expressed as mg of gallic acid equivalent (GAE) per g of sample (mg GAE /g).

2.5. Antioxidant activity

2.5.1. DPPH assay

For the DPPH method, a solution of 0.1 µmol /L of the radical were prepared in 80% (v/v) ethanol. Curves were prepared with five different dilutions of each extract in 80% ethanol. Then, 500 µL of each prepared sample dilution was transferred to test tubes and 3.5 mL of DPPH solution (0.1 µmol /L) was added. The absorbances of the mixtures were determined at 517 nm after 1 h of reaction. The antioxidant activity was calculated, using Trolox standard curves and the results were expressed as Trolox equivalent (µmol Trolox /g) (KIM et al., 2002).

2.5.2. ABTS assay

The ABTS free radical scavenging activity assay was performed according to Re et al. (1999), with modifications. The radical was prepared by adding a sodium persulfate solution (2.45 mmol /L) at a 1: 1 ratio to the ABTS solution at 7 mmol /L. After homogenization, the mixture was placed in a water bath at 50 °C for 15 min. Then, 1 mL of this mixture was diluted in 80% (v/v) ethyl alcohol to an absorbance of 0.700 ± 0.05 at 734 nm, followed by 3.5 mL of the ABTS solution and 0.5 mL of each extract. The analysis was conducted in a spectrophotometer Model UV-M519, and the spectrophotometric reading was taken after 7 min of reaction. The antioxidant activity was calculated using Trolox standard curves, and the results were expressed as Trolox equivalent (µmol Trolox /g).

2.6. Anthocyanin determination

Identification and quantification of anthocyanins (before and after in vitro gastrointestinal digestion) were carried out by UHPLC, with adaptations from a previous protocol (HELLSTRÖM; MATTILA; KARJALAINEN, 2013). The chromatographic separation was achieved using an LC Thermo Scientific Accela (diode array detector (DAD), autoinjector, and Accela pump) (Thermo Fisher Scientific, Austin, TX). Separation was performed on a Shim-pack VP-ODS (250 x 4.6 mm, with a particle size of 4.6 µm and a pore size of 12 nm) (Shimadzu, Kyoto, Japan). The mobile phase consisted of water acidified with 5% formic acid (A) and acetonitrile (B), with elution in a linear gradient of 0 – 5 min (100 – 95% A and 0 – 5% B), a linear gradient of 5 – 14 min (95 – 87% A and 5 – 13% B), isocratic 10 – 14 min (87% A and 13% B), a linear gradient of 14 – 15 min (87 – 95% A and 13 – 5% B) and isocratic 15 – 16 min (95% A and 5% B). The flow was 1000 µL min-1, and the injection volume was 5 µL (partial loop), with a temperature of 25 °C for the inlet and 35 °C for the column. Peaks were detected at 535 nm. The concentration of cyanidin-3-glucoside and cyanidin-3-rutinoside was calculated based on the area of each peak, at 535 nm, using a calibration curve. The calibration curves obtained for anthocyanin-3-glucoside and anthocyanin-3-rutinoside showed a linearity range between the concentrations of 0.001 mg /mL and 0.2 mg /mL. The equations of the straight line were obtained, being for anthocyanin-3-glucoside: y = 6E+07x - 46754, with R2 = 0.9931 and for anthocyanin-3-rutinoside: y = 6E+07x + 270072, with R2 = 0 ,9963.

2.7. Simulated gastrointestinal digestion

.All fermented açai samples were digested according with the standardized in vitro static digestion model previously reported by Minekus et al., (2014), with modifications. The method consists of submitting the extracts to specific conditions to simulate in vitro the gastric and intestinal phases. Thus, it would be possible to clarify the influence of human physiological conditions on the release and absorption of the compounds.

All assays were performed in 50 mL falcon tubes, kept in a thermostatic bath at 37 °C and agitation of 120 rpm. In the gastric phase (GP), 10 mL of liquid sample were homogenized with 7.5 mL of the GP electrolyte solution composed by 1.6 mL of

pepsin solution (25 000 U /mL), 5 mL of CaCl2 (0.3 M), 0.2 mL of HCl (1 M) to reach pH 3.0, and 0.695 mL of water. The samples were then incubated for 2 h.

Then, in the intestinal phase (IP), 20 mL of gastric chime (sample submitted to GP) was mixed with 11 mL of the IP electrolyte stock solution, comprising 5.0 mL of a 800 U /mL pancreatin solution, 2.5 mL of bile salts (160 mM), 40 mL of CaCl2 (0.3 M), 0.15 mL of NaOH (1 M) to reach pH 7.0, and 1.31 mL of water. This mixture was incubated for 2 h. After digestion, the samples were centrifuged for 20 min at 2850 g. The supernatant was frozen at -80 °C for further analysis of total phenolic compounds, cyanidin-3-glucoside, cyanidin-3-rutinoside, antioxidant capacity.

Bioaccessibility, which represents the percentage of compounds released from fermented açai, was expressed as a percentage and was determined as follow:

Bioaccessibility (%) = CA/CI x 100

where CA is the concentration of the compound in the intestinal phase and CI is the initial concentration of the compound in not digested fermented açai.

2.8. Cell viability and reactive oxygen species (ROS) generation assay

The extracts (SF 24h, LF 72h and AF 24h) that showed the highest total phenolic content after the simulated digestion test and their respective time 0 h were selected for testing in cell lines.

The cytotoxicity of the digested and not digested fermented açai extracts were evaluated on A549 (lung adenocarcinoma epithelial cells), HCT8 (human colon carcinoma) and IMR90 (normal human lung fibroblast). Briefly, cells were seeded into 96-well plates at a density of 6 × 103 cells /well (IMR90) and 1 × 104 cells /well (HCT8 and A549). Following the treatment (100, 500, 1000, and 2000 µg /mL) for 48 h, MTT (0.5 mg /mL) was added to each well, and the plates were incubated for 4 h at 37 °C. The formazan crystals were dissolved in DMSO and the absorbance was measured at 570 nm. The IC50 (50% cell viability inhibition), GI50 (50% growth inhibition), and LC50 (50% cell death) parameters were performed as described by (DO CARMO et al., 2018).

Intracellular reactive oxygen species (ROS) generation was assessed using the DCFH-DA assay. A549 cancer cells (6 × 104 per well) were treated for 1 h with

different digested and not digested fermented açai extracts (100 – 1000 µg /mL) or 22.5 µmol /L H_2O_2 (positive control) or culture medium (negative control). Following the treatment, H_2O_2 was added at 22.5 µmol /L in the wells and the fluorescence intensity (λ emission = 538 nm and λ excitation = 485 nm) was measured (ESCHER et al., 2018).

2.9. Mutagenicity evaluation

The A549 cancer cell line was plated in three 75 cm flasks at a density of 5 x 105 cells. The negative control group was treated with culture medium and the positive control received 4 µM cisplatin, in order to induce chromosomal aberrations. In the remaining groups the cells were treated only with fermented açai extract (250 µg /mL) and an association with cisplatin at 100, 250 e 500 µg /mL. For the chromosomal aberration test, we used the chromosomal break criterion described by Carmo et al. (2019). This criterion converts aberrations into interrupt events. Thus, gaps were not counted as chromosomal breakages; chromatic and chromosomal breaks were counted as single-breakage events; and ring chromosomes, dicentric chromosomes, tri- and tetra-radial figures, and complex rearrangements were counted as two-breakage events each. The chromosome breakage index (CBI) was determined by the average number of chromosome breaks observed per metaphase.

2.10. Statistical analysis

The analysis were performed in triplicate and the results were submitted to analysis of variance (ANOVA) using the statistical software R (version 4.0.2). The obtained means were compared by Tukey test at a 95% confidence level. Subsequently, linear and non-linear regressions were performed as a function of fermentation time, observing the best fit defined by the coefficient of determination (R^2) and p-value.

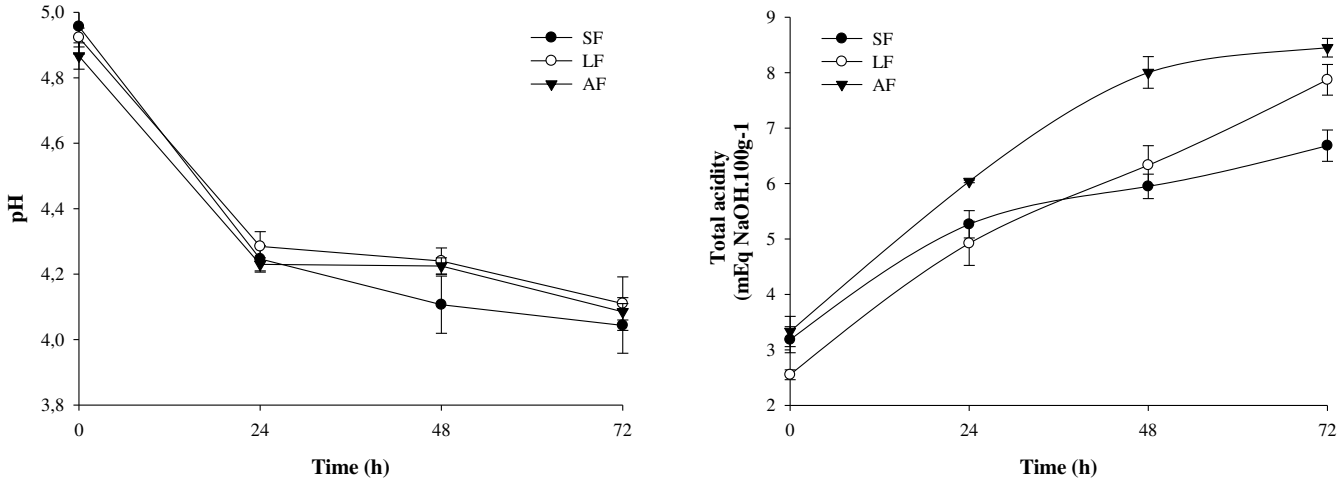
Data obtained from tests using cell lines were converted to percentage of negative control ± standard deviation. The data was statistically analyzed by two-way ANOVA followed by Tukey post hoc using Graphpad prism software, version 5.0 (Graphpad Prism software, USA). P values < 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Fermentation process

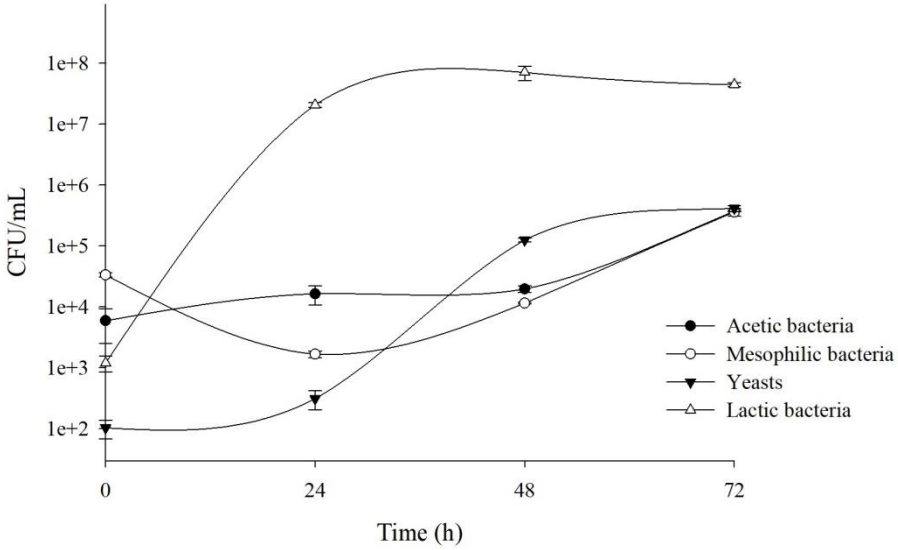
During spontaneous (SF), lactic (LF), and alcoholic (AF) fermentation of acai, the pH decreased mainly at the first 24 h, with subsequent stabilization (Figure. 1a), in parallel with the increase in the total acidity (Figure. 1b). Based on these results, the fermentation occurred as expected in a typical process with variation in acidity due to the production of organic acids from the metabolism of bacteria present in açai.

Figure 1. Total pH and acidity of medium during the spontaneous (SF), lactic (LF), and alcoholic (AF) fermentation of açai.



The spontaneous fermentation occurred via microbial succession, with a prevalence of lactic acid bacteria (LAB) growth in the first 24 h, reaching a final concentration of over 107 CFU /mL (Figure 2), with proportional raise in acidity (Figure 1b). After this time, the LAB population entered the stationary phase, explaining the lack of adjustment for the regression model for this bacterium. On the other hand, after 24 h it can be observed the development of the other populations present in the medium, mainly yeasts, followed by acetic bacteria. The metabolism of those populations was favored by the raise in acidity caused by the production of organic acids by LAB.

Figure 2. Bacteria and yeast counts during açai spontaneous fermentation.



Yeasts and acetic and mesophilic bacteria populations reached approximately 105 CFU /mL at the end of the process. The mathematical models found a non-significant lack of adjustment ($p>0.05$), with R2 above 90% for those populations. In general, the equations were well suited to the experimental data. Likewise, Aguiar et al., (2013) observed a predominance of LAB in the SF of açai within a closed system, in which the microaerobic environment favored metabolism.

3.2. Effect of fermentation on phenolic, anthocyanins, and antioxidant compounds of acai

The levels of total phenolics and anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) during açai fermentation, before and after simulated digestion are shown in Table 1. In addition, this same table shows the percentages of bioaccessibility, which refer to the amount of the compound in the fraction resulting from digestion in relation to the initial amount of the compound in the food before digestion.

Analyzing the fermentation process, it was observed that the AF led to the highest levels of total phenolics in all analyzed periods during açai fermentation; the mathematical model presents a point of maximum phenolic content at 36 h of fermentation. On the other hand, lactic fermentation generated extracts with the lowest total phenolic contents, independently of the fermentation time, with a decrease of 25.31% in the first 24 h and of 36.45% at the end of 72 h of fermentation (Table 1).

Although the total phenolics increased in AF, it was possible to observe that the cyanidin-3-glucoside content decreased in the first 24 h. This fact indicates the occurrence of the biotransformation of anthocyanins into lower-molecular weight phenolics, probably to for protocatechuic, caffeic, and ferulic acids, which are cited as the main phenolic compounds of cyanidin metabolism (Braga et al., 2018; Lang et al., 2023; Yuan et al., 2023). On the other hand, the cyanidin-3-rutinoside content remained stable during fermentation, indicating that this anthocyanin was not metabolized by the yeasts in the conditions of this experiment. Thus, it was not possible to fit an equation for this variable for AF.

In the first 24 h of fermentation, about 80% of the cyanidin-3-glucoside was consumed in the three fermentations. Some works mention that S. cerevisiae or Lactobacillus spp. strains that have low β-glucosidase activity produce fermented products with higher concentrations of anthocyanins compared to fermented products produced by microorganisms with high β-glucosidase activity (MULTARI et al., 2020; VERNOCCHI et al., 2011). This factor may have influenced the accelerated metabolism of cyanidin-3-glucoside, especially in the lactic fermentation in which it was not possible to detect the presence of this anthocyanin at the end of the fermentative process (72 h).

Another important factor was the addition of the inoculum that accelerated fermentation in the early phase, causing anthocyanins to be metabolized rapidly. The mathematical models show that biotransformation was slower in SF than in others. Likewise, Martín-Gómez et al., (2021) observed that the addition of inoculum can reduce fermentation time despite increasing the loss of phenolic compounds. At the end of the 72 h of fermentation, it was possible to observe decreases in the contents of phenolic compounds of 10.65%, 36.45%, and 13.11% for SF, LF, and AF, respectively.

Regarding the SF, the microbial succession profile is also clearly observed in these products. It is observed that in the first 24 h where fermentation is predominantly lactic, there is a reduction in total phenolics, as occurs during LF, which remains stable later when there is a predominance of yeast metabolism. Likewise, it was observed that anthocyanins reduced considerably during the first 24 hours with recovery when observing yeast growth in the fermentation medium.

Table 1 - Total phenolic and anthocyanin contents and bioaccessibility (BIO) of açai during fermentation before (BSD) and after (ASD) simulated digestion

Analysis	Time (h)	Spontaneous fermentation			Lactic fermentation			Alcoholic fermentation		
		BSD	ASD	BIO	BSD	ASD	BIO	BSD	ASD	BIO
Total phenolic (mg GAE.g^{-1})	0	23.28 ± 0.54^{a}	7.56 ± 0.53^{A}	32.47 %	21.37 ± 0.62^{a}	5.38 ± 0.02^{B}	25.17 %	24.70 ± 0.71^{a}	7.51 ± 0.57^{A}	30.40 %
	24	21.79± 0.33^{b}	11.48 ± 0.11^{A}	52.68 %	15.96± 0.64^{c}	4.44 ± 0.01^{C}	27.81 %	27.88 ± 0.57^{a}	6.95 ± 0.55^{B}	24.92 %
	48	20.71± 1.49^{b}	7.68 ± 0.19^{A}	37.08 %	16.41 ± 0.17^{c}	4.58 ± 0.02^{C}	27.91 %	26.89 ± 1.62^{a}	5.23 ± 0.26^{B}	19.44 %
	72	20.80 ± 0.10^{a}	5.99 ± 0.68^{B}	28.79 %	13.52 ± 1.14^{b}	8.72 ± 0.03^{A}	64.49 %	21.46 ± 0.73^{a}	5.74 ± 0.22^{B}	26.74 %
Equation		TP = 2331.86-8.4t +0.06t^2 R^2 =0.7020	TP = 805.26 +13.99t - 0.24t^2 R^2 =0.6720	N	TP = 2177.4-21.46t+0.14t^2 R^2 =0.8216	TP = 553.41 - 11.64t +0.22t^2 R^2 =0.9568	N	TP = 2469.5 +22.42t+0.7t^2 R^2 =0.6524	TP = 768.25 - 6.26t - 0.046t^2 R^2 =0.6793	N
Cyanidin-3-Glycoside (mg.g^{-1})	0	8.02±0.80^{a}	ND	-	7.36±0.56^{a}	ND	-	6.94±0.57^{a}	ND	-
	24	1.37±0.17^{a}	0.37±0.17	27.01 %	1.75±0.01^{a}	0.25±0.01	14.28 %	1.62±0.60^{a}	ND	-
	48	0.70±0.04^{ab}	ND	-	0.47±0.13^{b}	ND	-	1.12±0.17^{a}	ND	-
	72	0.62±0.18^{a}	ND	-	0.00±0.00^{b}	ND	-	0.9±0.10^{a}	ND	-

Equation		Cy3Glic = 7.75 -0.30t +0.002t^2 R^2 = 0.9536	N	N	Cy3Glic = 7.18 - 0.26t -0.002t^2 R^2 = 0.9771	N	N	Cy3Glic = 6.72- 0.36t -0.002t^2 R^2 = 0.9508	N	N
Cyanidin-3-Rutinoside (mg.g^{-1})	0	16.73±1.68^a	ND	-	15.36±1.18^a	ND	-	14.49±1.20^a	ND	-
	24	11.75±0.06^b	ND	-	13.25±0.53^a	0.05±0.05	0.37 %	14.86±3.21^a	ND	-
	48	12.37±0.73^a	ND	-	8.38±2.38^a	ND		12.34±3.21^a	ND	-
	72	11.62±0.18^a	ND	-	3.75±0.35^b	0.75±0.00	20.00 %	13.84±1.06^a	ND	-
Equation		Cya3Rut = 16.38 -0.19t +0.002t^2 R^2 = 0.7871	N	N	N	N	N	N	N	N

Data represent the mean ± SD (n = 3). In each line, different letters mean significant differences ($p < 0.05$), uppercase and lowercase letters must be evaluated separately.BSD (Before simulated digestion). ASD (After simulated digestion). BIO (% Bioaccessibility). N = It was not possible to adapt the mathematical model. ND (Below detection levels). TP (Total phenolic). Cy3Glic (Cyanidin-3-Glycoside). Cya3Rut (Cyanidin-3-Rutinoside)

Table 2 Antioxidant capacity of açai during fermentation before (BSD) and after (ASD) simulated digestion.

Analysis	Time (h)	Spontaneous fermentation		Lactic fermentation		Alcoholic fermentation	
		BSD	ASD	BSD	ASD	BSD	ASD
ABTS (µmol Trolox.g^{-1})	0	24.05±1.48^{a}	3.68±0.51^{B}	24.50±2.82^{a}	12.35±0.53^{A}	22.63±0.44^{a}	4.03±0.36^{B}
	24	28.55±2.20^{b}	6.39±0.17^{B}	12.13±0.12^{c}	7.49±0.12^{A}	35.46±0.28^{a}	4.55±0.07^{C}
	48	22.84±0.30^{a}	3.61±0.27^{B}	23.66±1.75^{a}	11.39±0.74^{A}	23.66±1.73^{a}	3.68±0.35^{B}
	72	23.98±0.01^{a}	4.35±0.33^{B}	17.77±2.16^{b}	20.29±1.29^{A}	19.09±1.08^{b}	3.59±0.31^{B}
Equation		N	N	N	ABTS =12.16-0.31t -0.005t^2 R^2 = 0.9556	ABTS =24.21 +0.45t -0.07t^2 R^2 = 0.6458	N
DPPH (µmol Trolox g^{-1})	0	26.35±0.36^{a}	4.00±0.09^{B}	24.05±4.56^{a}	10.57±0.91^{A}	28.60±0.07^{a}	5.50±0.51^{B}
	24	31.39±3.20^{a}	10.16±1.25^{A}	13.31±2.51^{b}	8.85±0.81^{A}	30.27±2.27^{a}	5.65±2.68^{A}
	48	29.81±0.69^{a}	8.72±0.62^{A}	24.99±1.13^{b}	7.74±0.61^{A}	28.26±0.01^{a}	3.31±0.75^{B}
	72	25.15±0.96^{a}	6.31±0.66^{B}	14.03±2.27^{b}	15.90±0.51^{A}	24.17±1.87^{a}	3.27±0.39^{C}
Equation		DPPH =26.52+0.28t-0.004t^2 R^2 =0.6672	DPPH = 4.33+0.29t -0.003t^2 R^2= 0.8117	N	DPPH =11.00 +0.24t -0.04t^2 R^2 = 0.8585	DPPH = 42.20+0.69t-0.01t^2 R2 =0.9852	N

Data represent the mean ± SD (n = 3). In each line, different letters mean significant differences ($p < 0.05$), uppercase and lowercase letters must be evaluated separately.BSD (Before simulated digestion). ASD (After simulated digestion). BIO (% Bioaccessibility). N = It was not possible to adapt the mathematical model. ND (Below detection levels).

Although there is an increase in the concentration of phenolics in AF, there is a decrease in bioaccessibility. On the other hand, the reduction of phenolic content in LF leads to a proportional increase in bioaccessibility. This is even observed in the microbial succession existing in SF.

In this study, two methods (ABTS and DPPH) were used to determine the antioxidant capacity of fermented açai (Table 2). In the first 24 h of AF, there was an increase in the antioxidant capacity, which was directly related to the biotransformation of anthocyanins into phenolic compounds of lower molecular weight (Braga, et al., 2018; Yuste et al., 2019). However, during the course of fermentation, both the antioxidant activity and the levels of anthocyanins and phenolic compounds were decreased. This can be explained by secondary reactions such as oxidation and condensation that occur during fermentation (Ávila et al., 2009; Braga, et al., 2018; Martín-Gómez et al., 2021; Yuste et al., 2019). It was not possible to establish a mathematical model that could explain the behavior of the ABTS variables in SF and LF, as well as DPPH in LF.

3.3. Bioaccessibility and effects of in vitro simulated digestion on phenolic, anthocyanins, and fermented acai capacity

The simulated digestion was used to assess the bioaccessibility of phenolic compounds and anthocyanins after gastric and intestinal passage. Thus, the results obtained here simulate the amount of the component that would be available for absorption.

Gastric and intestinal digestion drastically decreased the concentrations of phenolic compounds and anthocyanins, as well as the antioxidant capacity of all fermented products. The entire digestive process, including digestive enzymes, pH changes, microorganisms, saline solution concentration, and agitation contribute to the metabolism and degradation of phenolic compounds present in the food matrix. The same was observed in several studies with reductions ranging from 35% to 95% (CEBECI; ŞAHIN-YESILCUBUK, 2014; DA SILVA HAAS et al., 2019; GIACONIA et al., 2022; HAO et al., 2021; PHAN; BUCKNALL; ARCOT, 2019b; SENGUL; SUREK; NILUFER-ERDIL, 2014).

The results demonstrate that naturally fermented açai (SF) for 24 h showed greater bioaccessibility of phenolics (52.68%) and cyanidin-3-glucoside (27.01%) than its in natura equivalent (Table 1). In addition, it also showed greater antioxidant

capacity after simulated digestion (Table 2). These are promising results that demonstrate the importance of studying ways to increase the bioaccessibility of bioactive properties through processing, showing that one should consider not only the nutritional content of the food but also all that involves its digestion and absorption.

Lactic fermentation by 24 and 72 h increased the bioaccessibility of cyanidin-3-rutinoside compared to not fermented açai, in which it was not possible to detect anthocyanins after the digestion process. Another relevant result was the percentage of total phenolic bioaccessibility for LF 72 h (64.49%) compared to non-fermented acai (25.17%). It is interesting to note that despite having a lower concentration of phenolic and anthocyanins (before simulated digestion) LF 72h presented considerably larger bioaccessibility than the not fermented acai sample. That is, in this case, fermentation was really effective in increasing the bioaccessibility of total phenolic compounds and cyanidin-3-rutinoside (Table 1). Similarly, it was observed that antioxidant capacity was increased in lactic fermentation 72 h (after simulated digestion) compared to not fermented açai (Table 2). Other authors mention that the fermentation process can help obtain a product with greater antioxidant capacity and more bioaccessible compounds and generate for the end consumer better health benefits (GAO et al., 2022; VALERO-CASES; NUNCIO-JÁUREGUI; FRUTOS, 2017).

3.4. In vitro cell viability and intracellular antioxidant activity by measurement of reactive oxygen species (ROS)

The beneficial effects attributed to açai are directly linked to its content of phenolic compounds (ARANHA et al., 2019; MACHADO et al., 2019; SONG et al., 2021). Thus, the initial time (0 h) of each fermentation and the time with the highest concentration of phenolic compounds after simulated digestion (SF 24 h, LF 72 h, or AF 24 h) were selected for analysis.

Regarding the cell viability assay (Figure 3 a-b), the HCT8 cells, appeared to be more sensitive to SF O h sample, which is basically açai in natura, showing a 50% reduction in the cell growth index (GI) at 1438 mg /g before and after simulated digestion. This effect can be attributed to the cyanidin-3-glycoside (8.02±0.80 mg /g) and cyanidin-3-rutinoside (16.73±1.68 mg /g) contents in SF O h. In this sense, Cvorovic et al. (2010) demonstrated that cyanidin exhibited a cytotoxic effect on

LoVo (human colorectal adenocarcinoma metastasis) and LoVo/ADR (doxorubicin-resistant metastatic human colorectal adenocarcinoma) cells with IC50 of 46.9 ± 1.8 µM and 26.2 ± 6.2 µM respectively.

In general, the cell exposure to different concentrations of fermented açai extracts before digestion and after simulated digestion exhibited no cytotoxic effects, whereas they did not cause cell death, reduce viability, or inhibit cell growth, in both cancer and normal cells (Figures 3 a-b). Similarly, Silva et al., (2014) evaluated açai extracts and did not observe cytotoxicity in colorectal adenocarcinoma cell lines (CACO-2 and HT-29) or in breast cancer cell lines (MDA-MB-468).

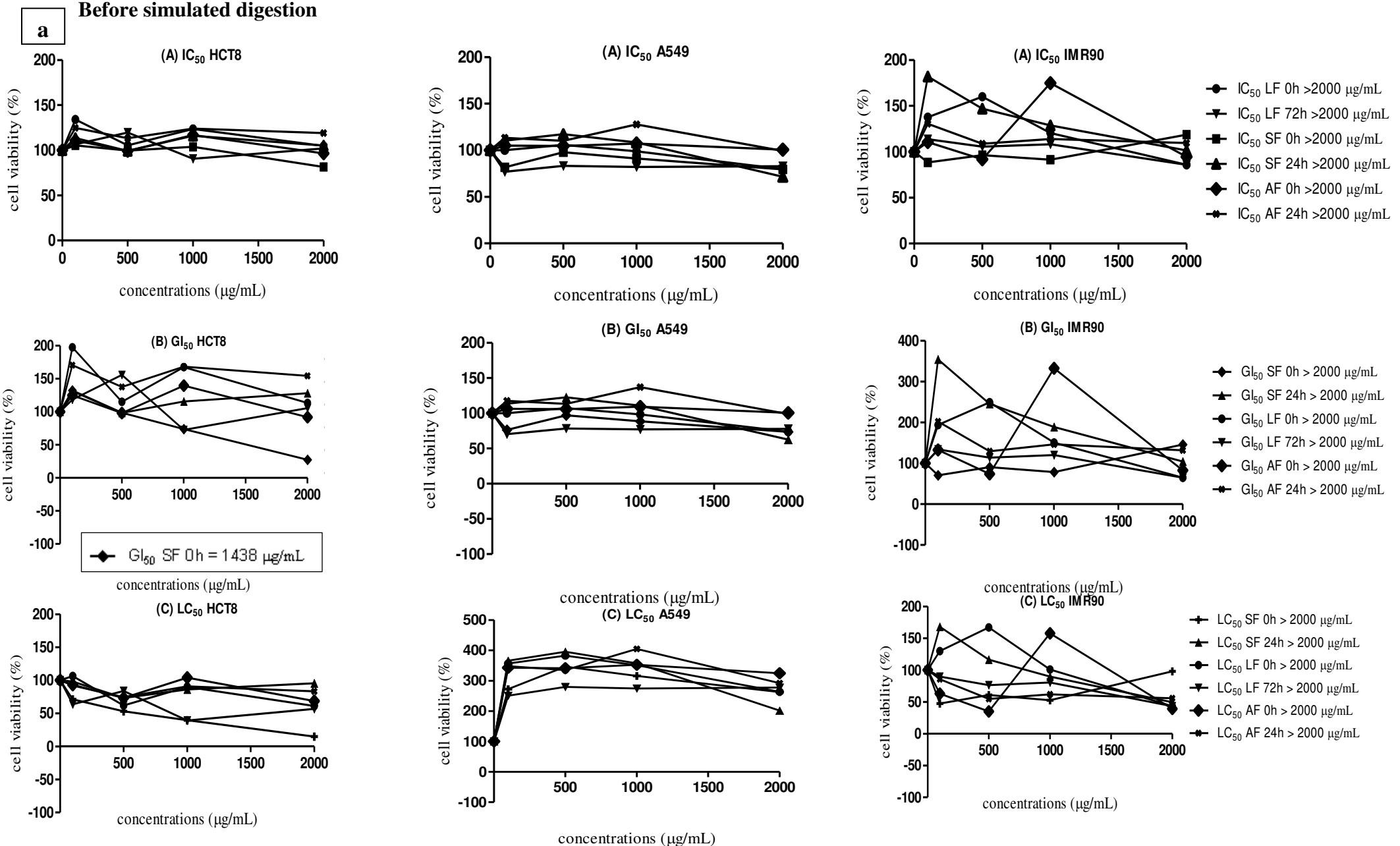
Fermented açai extracts, before and after simulated digestion, were evaluated for intracellular generation of ROS in the HCT8 cell line (Figure. 4 a-b). The first four bars of the graph refer to how much the sample is able to induce the formation of intracellular reactive oxygen species (ROS), while the last three bars of the graph refer to the ability of the sample to protect cells from the production of ROS caused by hydrogen peroxide. Therefore, in general, the use of isolate açai does not induce the generation of ROS.

The results showed that before digestion LF induced ROS formation while AF 0 h (100 and 500 µg /mL) potentiated ROS production when added together with hydrogen peroxide. In contrast, 24h AF showed a protective effect on cells, reducing reactive oxygen species generated by H2O2 to levels similar to the control. The observed antioxidant action may be related to the high levels of cyanidin-3-glucoside (1.62 ± 0.60 mg /g) and cyanidin-3-rutinoside (14.86 ± 3.21 mg /g). The in vitro antioxidant potential of these specific compounds has already been demonstrated in other studies (BAO et al., 2019b; SASIKUMAR; JYOTI DAS; CHANDRA DEKA, 2021).

Overall, after the digestion simulation, none of the fermented products induced the formation of ROS. Conversely, AF 0 h and 24 h showed antioxidant activity since they reduced peroxide-induced ROS formation. This intracellular antioxidant effect can be attributed to the phenolic compounds formed during fermentation and simulated digestion from the metabolism and degradation of anthocyanins. It is known that the main products of degradation and metabolism of cyanidin's are protocatechuic acid, caffeic acid, and ferulic acid, which act as potent antioxidants (Braga et al., 2018; Giaconia et al., 2022; Hornedo-Ortega et al., 2016; Yuan et al., 2023).

Figure 3. Cytotoxicity and proliferation inhibition of cell lines A549, HCT8 and IMR90 of fermented açai before (a) and after (b) simulated digestion. A) IC50; B) IG50; and C) LC50.

Before simulated digestion



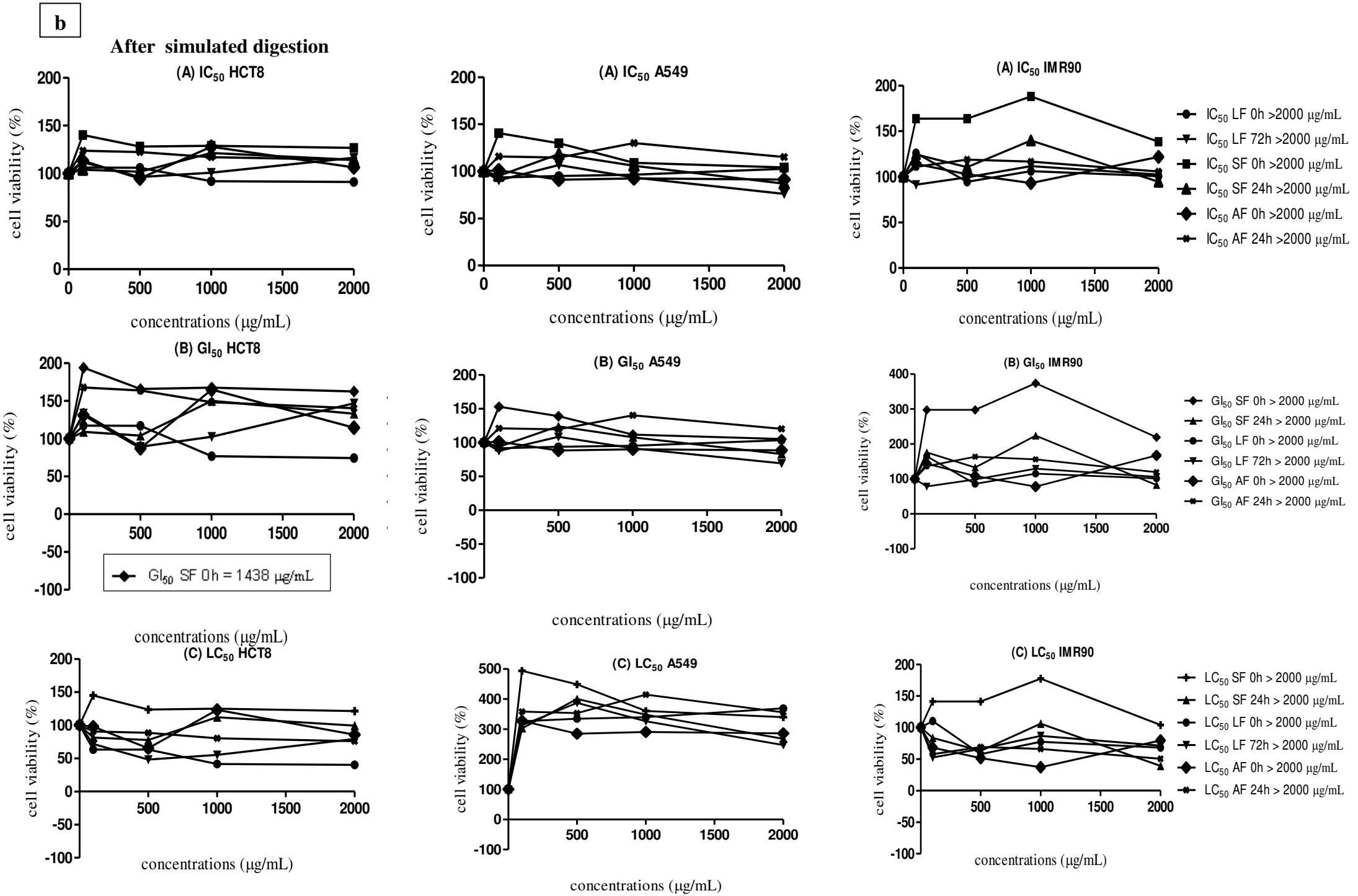
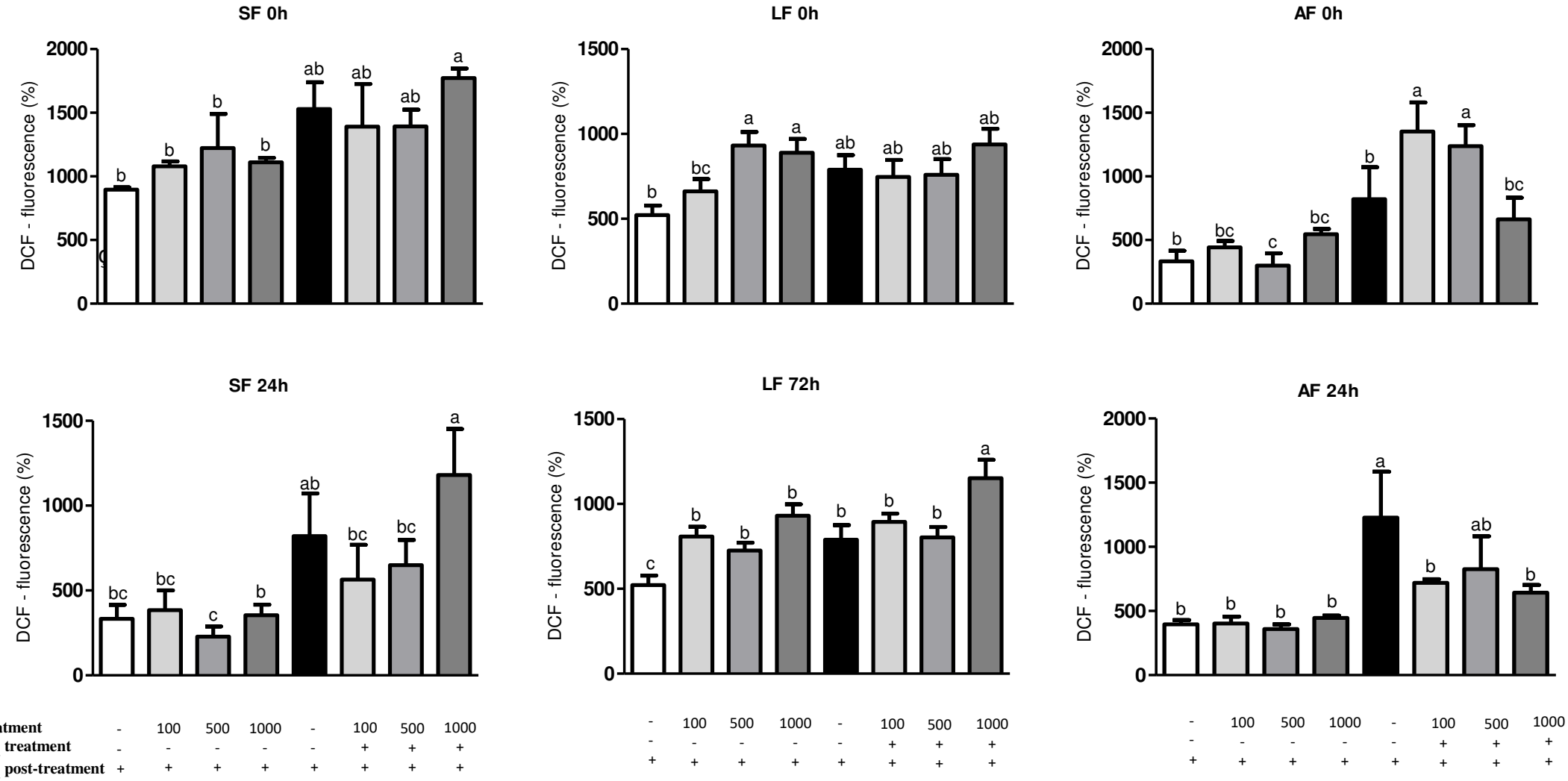
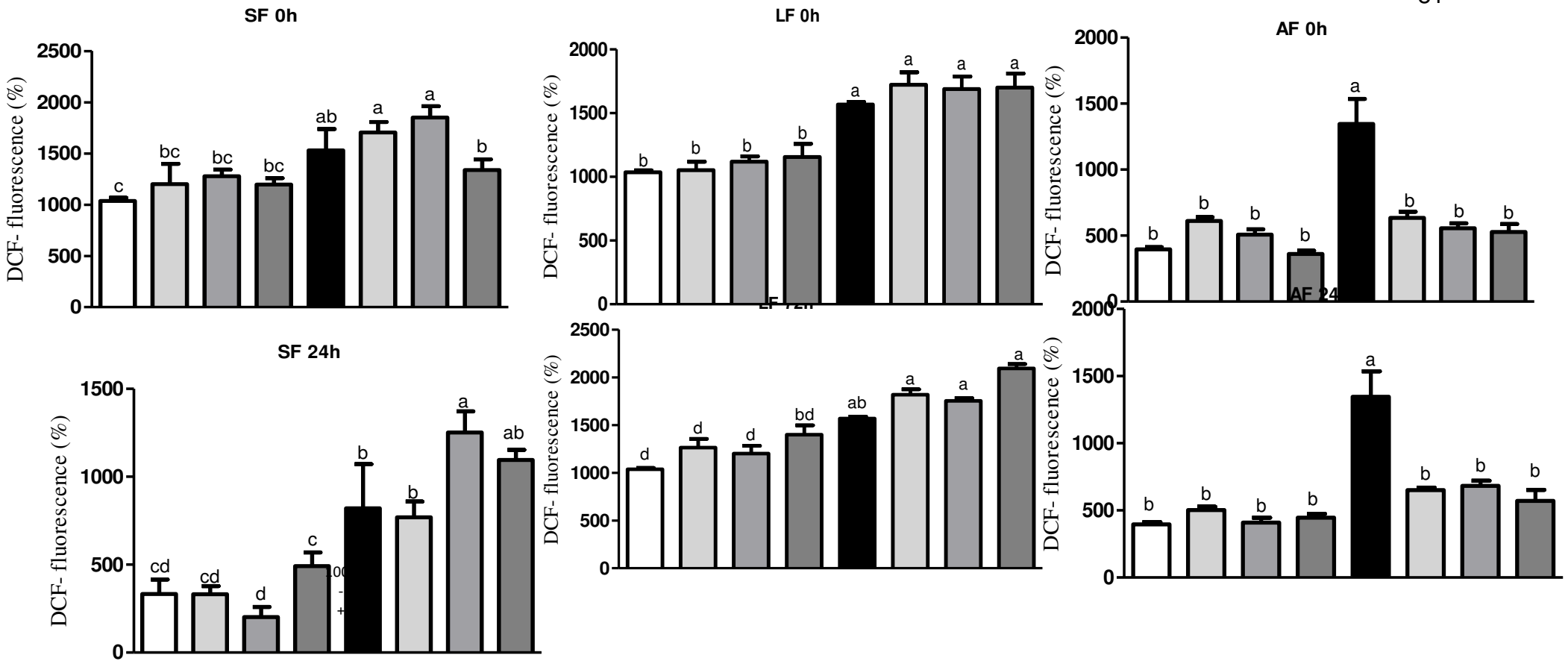

b
After simulated digestion
(A) IC50 HCT8
(A) IC50 A549
(A) IC50 IMR90
cell viability (%)
concentrations (µg/mL)
IC50 LF 0h >2000 µg/mL
IC50 LF 72h >2000 µg/mL
IC50 SF 0h >2000 µg/mL
IC50 SF 24h >2000 µg/mL
IC50 AF 0h >2000 µg/mL
IC50 AF 24h >2000 µg/mL
(B) GI50 HCT8
(B) GI50 A549
(B) GI50 IMR90
GI50 SF 0h = 1438 µg/mL
GI50 SF 0h > 2000 µg/mL
GI50 SF 24h > 2000 µg/mL
GI50 LF 0h > 2000 µg/mL
GI50 LF 72h > 2000 µg/mL
GI50 AF 0h > 2000 µg/mL
GI50 AF 24h > 2000 µg/mL
(C) LC50 HCT8
(C) LC50 A549
(C) LC50 IMR90
LC50 SF 0h > 2000 µg/mL
LC50 SF 24h > 2000 µg/mL
LC50 LF 0h > 2000 µg/mL
LC50 LF 72h > 2000 µg/mL
LC50 AF 0h > 2000 µg/mL
LC50 AF 24h > 2000 µg/mL

Figure 4. Concentration of Intracellular Reactive Oxigen Species (ROS) in HCT8 cells treated with extracts of acai subjected to spontaneous (SF), lactic (LF) or alcoholic (AF) fermentation before (a) and after (b) simulated digestion.

a



b


SF 0h
LF 0h
AF 0h
SF 24h
LF 72h
AF 24
DCF- fluorescence (%)
Treatment - 100 500 - 100 500 1000
H202 treatment - - - - + + +
H202 post-treatment + + + + + + +

3.5. Mutagenicity evaluation

This analysis evaluates whether the sample has a protective potential against chromosomal damage induced by cisplatin in A549 cells. Cisplatin is a drug that induces an increase in chromosomal aberrations that is directly related to its mutagenic effect (CARMO et al., 2019).

The results demonstrate that cisplatin was able to significantly induce levels of chromosomal aberrations when compared to the negative control (Figure. 5 a-b). The extract AF 24 h did not induce the levels of chromosomal aberrations. Furthermore, the concentration of 250 µg/g of the fermented açai extract was able to reduce by 39.40% the chromosomal aberrations caused by cisplatin in A549 cells. These results suggest that this specific concentration of AF 24 has in vitro antimutagenic potential, which can be related to its in vitro antioxidant effect due to the presence of phenolic compounds (27.88 ± 0.57 mg /g) (CARMO et al., 2019; GAO et al., 2013; KHANDUJA et al., 2006) .

4. CONCLUSION

Açai is a natural source of bioactive compounds, mainly anthocyanins, and its consumption can bring health benefits. However, not all components present are bioaccessible to be absorbed. In this work, we showed that both SF and LF increased the bioaccessibility of phenolic compounds and antioxidant activity. Moreover, in in vitro assays, AF extract presents intracellular antioxidant activity, antimutagenic effect and no cytotoxicity.

To the best of our knowledge, this is the first study that evaluated different açai fermentation times on the bioaccessibility of phenolic compounds after simulated digestion. Thus, this study brings important information that can be used in production, since spontaneously fermented açai is already a food consumed locally in the producing regions of this fruit.

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GENERAL CONCLUSION

In general, anthocyanins are quite unstable, this also occurs during the gastrointestinal digestion process. Therefore, studying strategies to improve the bioavailability of these compounds becomes crucial to enhance their health benefits. The comprehensive review highlighted that, when facing challenges such as pH variations and enzymatic action in the digestive tract, microencapsulation emerges as an effective tool to preserve the integrity of anthocyanins, resulting in a general improvement in bioaccessibility *in vitro,* however *in vivo* studies are needed to prove the real effectiveness of this strategy. Co-ingestion is another tool that should be further explored, as the interaction of anthocyanins with other food components can significantly influence their absorption and use by the body. Future research can direct efforts to investigate how strategic food combining can optimize anthocyanin bioavailability.

Furthermore, the results of the second study provide evidence that specific strategies, such as spontaneous fermentation and lactic acid fermentation, can significantly increase the bioaccessibility of phenolics and cyanidin glycosides. The absence of cytotoxicity in the cell lines tested and the antioxidant and antimutagenic effects observed in alcoholic fermentation highlight the potential health benefits of these fermented products.

These findings not only emphasize the strategic importance of microencapsulation, processing, co-ingestion, and fermentation but also point to the continued need for research exploring different raw materials and processes. In addition, this study significantly contributes to the advancement of the development of functional foods, highlighting promising ways to improve the effective delivery of anthocyanins to the body.