UNIVERSITAT POLITÈCNICA DE VALÈNCIA

INSTITUTO UNIVERSITARIO DE INGENIERÍA DE ALIMENTOS – FoodUPV

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Caracterización fisicoquímica, tecnológica y funcional del residuo procedente de la obtención de la bebida vegetal de almendra. Estrategias de valorización.

TESIS DOCTORAL

Presentada por:
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Directoras:
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Febrero 2024

La Dra. Noelia Betoret Valls, profesora titular del Departamento de Tecnología de Alimentos de la Universitat Politècnica de València,

Y la Dra. María Ester Betoret Valls, investigador contratado doctor del Instituto Universitario de Ingeniería de Alimentos - FoodUPV de la Universitat Politècnica de València,

CONSIDERAN: que la memoria titulada "Caracterización fisicoquímica, tecnológica y funcional del residuo procedente de la obtención de la bebida vegetal de almendra. Estrategias de valorización" que presenta D. Stevens Duarte Serna, para aspirar al grado Doctor por la Universitat Politècnica de València, y que ha sido realizada bajo su dirección en el Instituto Universitario de Ingeniería de Alimentos - FoodUPV de la Universitat Politècnica de València, reúne las condiciones adecuadas para constituir su tesis doctoral, por lo que AUTORIZAN al interesado para su presentación.

Valencia, febrero de 2024

Fdo.: Noelia Betoret Valls

Fdo.: María Ester Betoret Valls

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PRÓLOGO

Justificación del estudio

El mercado de las bebidas vegetales está en continuo crecimiento debido a la alta demanda de productos de origen vegetal. En España el consumo de bebidas vegetales fue de 244.676,52 L en 2022 (STATISTA 2022). Las bebidas obtenidas a partir de cereales, frutos secos y oleaginosas se encuentran entre las más populares en el mercado por sus propiedades funcionales. Algunas de las materias primas más utilizadas para la elaboración de bebidas vegetales incluyen arroz, avena, chufa, quinoa, nueces y almendra. De todas ellas, la almendra, que pertenece al grupo de las oleaginosas, destaca por ser una de las más demandadas en el mercado debido al alto contenido en compuestos fenólicos y a sus propiedades antioxidantes (Bolling, 2017). El proceso para la elaboración de estas bebidas consiste en una extracción sobre la materia prima triturada y utilizando agua como disolvente. Posteriormente, la fase líquida se somete a operaciones de homogenización, pasteurización y rectificación antes de su comercialización. La fase sólida que se obtiene de la extracción se le denomina torta o bagazo y suele ser desechada o utilizada para alimentación animal. Sin embargo, este bagazo, en el caso de la almendra, contiene una cantidad considerable de compuestos nutricionales que hacen muy interesante su revalorización como ingrediente funcional para la industria alimentaria.

Hoy en día, la generación masiva de residuos y subproductos por parte de la industria alimentaria es uno de los mayores problemas que se enfrentan a nivel mundial. Según la Comisión Europea, a nivel global, se desperdicia aproximadamente un 13% de la producción, equivalente a 366 millones de toneladas dentro de la Unión Europea (FAO, 2022). España, específicamente, descarta alrededor de 7,5 millones de toneladas de alimentos al año. La industria alimentaria está trabajando, desde

hace años, para implementar los objetivos de desarrollo sostenible (ODS) aprobados por las Naciones Unidas en 2015 como parte de la Agenda 2030. Particularmente, el ODS 12 vela por la producción responsable y sostenible promoviendo acciones dirigidas a la prevención del desperdicio de alimentos, la revalorización de residuos y la economía circular.

En este contexto, en la presente tesis doctoral se realiza una caracterización fisicoquímica, tecnológica y funcional, y se estudia la posibilidad de revalorizar el subproducto resultante del proceso de obtención de la bebida vegetal de almendra. Se considera la posibilidad de realizar un aprovechamiento integral, sin el uso de disolventes ni la generación de efluentes, y mediante tecnologías disponibles que permitan mantener en la medida de lo posible un adecuado perfil nutricional. Se realiza una aproximación tecnológica que evalúa tanto las propiedades tecnológicas y de estabilidad del producto final como su funcionalidad, definida tanto por la composición en componentes bioactivos del producto final como por el efecto de estos sobre la microbiota intestinal. Se considera, además, una aplicación interesante desde el punto de vista industrial y de mercado.

RESUMEN

El mercado de bebidas vegetales está experimentando un crecimiento constante debido a la creciente demanda de productos de origen vegetal. Dentro de este mercado, la bebida de almendra, perteneciente al grupo de las oleaginosas, destaca por su alto contenido en nutrientes, compuestos fenólicos y sus propiedades antioxidantes. Por otra parte, la generación masiva de residuos y subproductos por parte de la industria alimentaria representa uno de los mayores desafíos a nivel global. De acuerdo con la Comisión Europea, aproximadamente se desperdicia un 13% de la producción alimentaria mundial, equivalente a 366 millones de toneladas dentro de la Unión Europea. La industria alimentaria ha estado trabajando en la implementación de los Objetivos de Desarrollo Sostenible (ODS) adoptados por las Naciones Unidas en 2015, con especial atención en el ODS 12, que promueve la producción responsable y sostenible. Este objetivo busca prevenir el desperdicio de alimentos, revalorizar residuos y promover la economía circular como parte de la Agenda 2030.

En este contexto, el objetivo general de la presente tesis fue estudiar las posibilidades de revalorización del subproducto resultante del proceso de obtención de la bebida vegetal de almendra. Determinar las propiedades fisicoquímicas, tecnológicas y funcionales de la materia prima. Evaluar el efecto de la deshidratación (secado con aire caliente y liofilización) sobre las propiedades fisicoquímicas, el contenido de componentes bioactivos, su bioaccesibilidad y su influencia sobre la microbiota. Finalmente, se consideró la posibilidad de obtener un producto deshidratado con probióticos.

La consecución de este objetivo se abordó desde tres enfoques que se presentan en tres capítulos en los que se ha estructurado el apartado de resultados. En el

primer capítulo se evaluó el impacto del secado por aire caliente a 60 °C y 70 °C y de la liofilización, sobre las propiedades tecno-funcionales del bagazo de almendra. Se analizaron las curvas de secado y las isotermas de sorción. Luego, se evaluó el efecto del almacenamiento a temperatura ambiente y en condiciones aceleradas de los polvos obtenidos por ambos métodos de secado; durante 6 meses, se monitoreó el crecimiento microbiológico, la acidez, el índice de peróxidos, la capacidad antioxidante y el contenido de polifenoles. Finalmente, se evaluó la idoneidad del polvo de bagazo de almendra como sustituto en la elaboración de productos de panadería, concretamente galletas.

Tanto el secado por aire caliente como la liofilización resultaron ser operaciones adecuadas para estabilizar el bagazo de almendra. Ambos métodos de secado, combinados con un triturado adecuado, proporcionaron polvos con propiedades favorables para su uso en la industria alimentaria. En relación con la actividad antirradical, no se presentaron diferencias significativas entre las muestras deshidratadas, si bien el contenido en fenoles totales fue mayor en las muestras liofilizadas. En cuanto al almacenamiento durante 6 meses, al finalizar dicho periodo se observó un aumento en la capacidad antirradical, así como en el contenido de fenoles totales, especialmente notable en las muestras secadas por aire caliente y sometidas a almacenamiento acelerado. Los valores de acidez e índice de peróxido aumentaron considerablemente durante el almacenamiento acelerado, posiblemente debido a la descomposición de los ácidos grasos. Finalmente, se evaluó la idoneidad del polvo de bagazo de almendra como ingrediente sustitutivo en la elaboración de productos de panadería. Los resultados mostraron la idoneidad del subproducto para ser utilizado como sustituto de la harina de trigo en la elaboración de galletas ya que proporcionó un producto de textura adecuada con mayor contenido en fibra y componentes con capacidad antirradical.

El segundo capítulo comprende los estudios relacionados con la influencia del proceso de deshidratación en la digestión gastrointestinal *in vitro* (fase oral, gástrica e intestinal) y en la fermentación colónica, con especial atención sobre los compuestos bioactivos específicos, microbiota fermentativa del colon y los ácidos grasos de cadena corta. La digestión gastrointestinal *in vitro* redujo la cantidad de compuestos polifenólicos específicos. En cuanto a los fenoles totales y la capacidad antioxidante, aumentaron progresivamente durante las etapas gástrica, intestinal y colónica. Los sustratos afectaron la estructura de la comunidad microbiana fermentativa del colon, siendo significativamente diferente en las muestras control. La fermentación colónica favoreció el crecimiento de bacterias productoras de ácidos grasos de cadena corta, predominando los ácidos grasos de cadena ramificada debido al alto contenido de proteína del bagazo de almendra.

Finalmente, en el tercer capítulo, se aborda una revisión general sobre cómo las operaciones de procesado de alimentos y la digestión gastrointestinal *in vitro* afectan la viabilidad de células probióticas en alimentos no lácteos. Se analizan diversos factores de estrés que afectan la viabilidad de los microorganismos probióticos, se detallan estrategias para incrementar las células viables en alimentos probióticos no lácteos y se describen los mecanismos que fomentan los cambios en estos microorganismos para mejorar su supervivencia. Además, se presenta un estudio de caso enfocado en determinar el efecto de la incorporación de *Lactobacillus salivarius* spp al bagazo de almendra fresco. Este sustrato fue utilizado para el crecimiento del probiótico y se evaluó el impacto de la matriz de incubación y las altas presiones de homogeneización (HPH) en su crecimiento, hidrofobicidad y resistencia al secado por aire caliente y la digestión *in vitro*. Los resultados muestran que el bagazo de almendra conserva nutrientes y es adecuado para el crecimiento de células de *Lactobacillus*. El probiótico demostró crecimiento en todas las condiciones

estudiadas y sobrevivió a la digestión *in vitro*, con mejores resultados cuando fue encapsulado por HPH, mejorando su resistencia a condiciones adversas como el secado por aire caliente y la digestión gastrointestinal *in vitro*.

ABSTRACT

The vegetable beverage market is experiencing steady growth due to the increasing demand for products of vegetable origin. Within this market, the almond beverage, which belongs to the oilseed group, stands out for its high content of nutrients, phenolic compounds and antioxidant properties. On the other hand, the massive generation of waste and by-products by the food industry represents one of the major global challenges. According to the European Commission, approximately 13% of the world's food production is wasted, equivalent to 366 million tonnes within the European Union. The food industry has been working on the implementation of the Sustainable Development Goals (SDGs) adopted by the United Nations in 2015, with a particular focus on SDG 12, which promotes responsible and sustainable production. This goal aims to prevent food waste, revalue waste and promote the circular economy as part of the 2030 Agenda.

In this context, the general objective of this thesis was to study the possibilities of revaluing the by-product resulting from the process of obtaining almond plant-based beverage. To determine the physicochemical, technological and functional properties of the raw material. Evaluate the effect of dehydration (hot air drying and freeze-drying) on the physicochemical properties, the content of bioactive components, their bioaccessibility and their influence on the microbiota. Finally, the possibility of obtaining a dehydrated product with probiotics was considered.

The achievement of this objective was approached through three perspectives presented in three chapters, structuring the results section accordingly. In the first chapter, the impact of hot air drying at 60 °C and 70 °C and freeze-drying on the techno-functional properties of almond bagasse was evaluated. Drying curves and sorption isotherms were analysed. Then, the effect of storage at room temperature

and under accelerated conditions of the obtained powders by both drying methods was evaluated; over a period of 6 months, microbiological growth, acidity, peroxide index, antioxidant capacity, and polyphenol content were monitored. Finally, the suitability of almond bagasse powder as a substitute in the production of bakery products, specifically biscuits, was assessed.

Both hot air drying and freeze drying proved to be suitable operations to stabilise almond bagasse. Both drying methods, combined with appropriate grinding, provided powders with favourable properties for use in the food industry. Regarding the antiradical activity, there were no significant differences between the dehydrated samples, although the total phenol content was higher in the freeze-dried samples. After a 6-month storage period, an increase in antiradical capacity and total phenolic content was observed, particularly noticeable in the samples dried by hot air and subjected to accelerated storage. Acidity and peroxide index values increased considerably during accelerated storage, possibly due to the decomposition of fatty acids. Finally, the suitability of almond bagasse powder as a substitute ingredient in the production of bakery products was evaluated. The results indicated its suitability as a substitute for wheat flour in the production of biscuits, providing a product with appropriate texture, increased fibre content, and components with antiradical capacity.

The second chapter comprises studies related to the influence of the dehydration process on *in vitro* gastrointestinal digestion (oral, gastric and intestinal phase) and colonic fermentation, with special attention on specific bioactive compounds, colonic fermentative microbiota and short-chain fatty acids. *In vitro* gastrointestinal digestion reduced the amount of specific polyphenolic compounds. As for total phenols and antioxidant capacity, they increased progressively during the gastric,

intestinal, and colonic stages. The substrates affected the structure of the colonic fermentative microbial community, being significantly different in the control samples. Colonic fermentation favoured the growth of short-chain fatty acid producing bacteria, with branched-chain fatty acids predominating due to the high protein content of almond bagasse.

Finally, the third chapter provides an overview of how food processing operations and *in vitro* gastrointestinal digestion affect the viability of probiotic cells in non-dairy foods. It discusses various stress factors that affect the viability of probiotic microorganisms, details strategies to increase viable cells in non-dairy probiotic foods and describes the mechanisms that promote changes in these microorganisms to improve their survival. In addition, a case study focused on determining the effect of incorporating *Lactobacillus salivarius* spp. into fresh almond bagasse is presented. This substrate was used for the growth of the probiotic and the impact of the incubation matrix and high homogenisation pressures (HPH) on its growth, hydrophobicity and resistance to hot air drying and *in vitro* digestion was evaluated. The results show that almond bagasse retains nutrients and is suitable for *Lactobacillus* cell growth. The probiotic showed growth in all conditions studied and survived *in vitro* digestion, with better results when encapsulated by HPH, improving its resistance to adverse conditions such as hot air drying and *in vitro* gastrointestinal digestion.

RESUM

El mercat de begudes vegetals està experimentant un creixement constant a causa de la demanda creixent de productes d'origen vegetal. Dins aquest mercat, la beguda d'ametlla, pertanyent al grup de les oleaginoses, destaca pel seu alt contingut en nutrients, compostos fenòlics i propietats antioxidants. D'altra banda, la generació massiva de residus i subproductes per part de la indústria alimentària representa un dels desafiaments més grans a nivell global. D'acord amb la Comissió Europea, aproximadament es desaprofita un 13% de la producció alimentària mundial, equivalent a 366 milions de tones dins de la Unió Europea. La indústria alimentària ha estat treballant en la implementació dels Objectius de Desenvolupament Sostenible (ODS) adoptats per les Nacions Unides el 2015, amb especial atenció a l'ODS 12, que promou la producció responsable i sostenible. Aquest objectiu cerca prevenir el malbaratament d'aliments, revalorar residus i promoure l'economia circular com a part de l'Agenda 2030.

En aquest context, l'objectiu general de la present tesi va ser estudiar les possibilitats de revaloració del subproducte resultant del procés d'obtenció de la beguda vegetal d'ametlla. Determinar les propietats fisicoquímiques, tecnològiques i funcionals de la matèria primera. Avaluar l'efecte de la deshidratació (assecat amb aire calent i liofilització) sobre les propietats fisicoquímiques, el contingut de components bioactius, la bioaccessibilitat i la influència sobre la microbiota. Finalment, es va considerar la possibilitat d'obtenir un producte deshidratat amb probiòtics.

La consecució d'aquest objectiu es va abordar des de tres enfocaments que es presenten en tres capítols en el que s'ha estructurat l'apartat de resultats. En el primer capítol es va avaluar l'impacte de l'assecat per aire calent a 60 °C i 70 °C, i de

la liofilització, sobre les propietats tecno-funcionals del bagàs d'ametlla. Es van analitzar les corbes d'assecat i les isotermes de sorció. Després, es va avaluar l'efecte de l'emmagatzematge a temperatura ambient i en condicions accelerades de les pols obtingudes pels dos mètodes d'assecat; durant 6 mesos, es va monitoritzar el creixement microbiològic, l'acidesa, l'índex de peròxids, la capacitat antioxidant i el contingut de polifenols. Finalment, es va avaluar la idoneïtat de la pols de bagàs d'ametlla com a substitut en l'elaboració de productes enfornats, concretament galetes.

Tant l'assecat per aire calent com la liofilització van resultar ser operacions adequades per estabilitzar el bagàs d'ametlla. Ambdós mètodes d'assecat, combinats amb un triturat adequat, van proporcionar pols amb propietats favorables per al seu ús a la indústria alimentària. En relació amb l'activitat antirradical, no es van presentar diferències significatives entre les mostres deshidratades, si bé el contingut en fenols totals va ser més gran en les mostres liofilitzades. En relació amb l'emmagatzematge durant 6 mesos, en finalitzar aquest període es va observar un augment en la capacitat antirradical, així com en el contingut de fenols totals, especialment notable a les mostres assecades per aire calent i sotmeses a emmagatzematge accelerat. Els valors d'acidesa i índex de peròxid van augmentar considerablement durant l'emmagatzematge accelerat, possiblement degut a la descomposició dels àcids grassos. Finalment, es va avaluar la idoneïtat de la pols de bagàs d'ametlla com a ingredient substitutiu en l'elaboració de productes enfornats. Els resultats van mostrar la idoneïtat del subproducte per ser utilitzat com a substitut de la farina de blat en l'elaboració de galetes ja que va proporcionar un producte de textura adequada amb més contingut en fibra i components amb capacitat antirradical.

El segon capítol comprèn els estudis relacionats amb la influència del procés de deshidratació a la digestió gastrointestinal *in vitro* (fase oral, gàstrica i intestinal) i a la fermentació colònica, amb especial atenció sobre els compostos bioactius específics, microbiota fermentativa del còlon i els àcids grassos de cadena curta. La digestió gastrointestinal *in vitro* va reduir la quantitat de compostos polifenòlics específics possiblement a la interacció entre compostos bioactius i macromolècules. Pel que fa als fenols totals i la capacitat antioxidant, van augmentar progressivament durant les etapes gàstrica, intestinal i colònica. Els substrats van afectar l'estructura de la comunitat microbiana fermentativa del còlon, sent significativament diferent a les mostres control. La fermentació colònica va afavorir el creixement de bacteris productors d'àcids grassos de cadena curta, predominant els àcids grassos de cadena ramificada a causa de l'alt contingut de proteïna del bagàs d'ametlla.

Finalment, al tercer capítol, s'aborda una revisió general sobre com les operacions de processament d'aliments i la digestió gastrointestinal *in vitro* afecten la viabilitat de cèl·lules probiòtiques en aliments no lactis. S'analitzen diversos factors d'estrès que afecten la viabilitat dels microorganismes probiòtics, es detallen estratègies per incrementar les cèl·lules viables en aliments probiòtics no lactis i es descriuen els mecanismes que fomenten els canvis en aquests microorganismes per millorar-ne la supervivència. A més, es presenta un estudi de cas enfocat a determinar l'efecte de la incorporació de *Lactobacillus salivarius* spp al bagàs d'ametlla fresca. Aquest substrat va ser utilitzat per al creixement del probiòtic i es va avaluar l'impacte de la matriu d'incubació i les altes pressions d'homogeneïtzació (HPH) en el creixement, la hidrofobicitat i la resistència a l'assecat per aire calent i la digestió *in vitro*. Els resultats mostren que el bagàs d'ametlla conserva nutrients i és adequat per al creixement de cèl·lules de *Lactobacillus*. El probiòtic va demostrar creixement en totes les condicions estudiades i va sobreviure a la digestió *in vitro*, amb millors

resultats quan va ser encapsulat per HPH, millorant la seva resistència a condicions adverses com l'assecat per aire calent i la digestió gastrointestinal *in vitro*.

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2 - Opportunities for the valorization of waste generated by the plant-based milk substitutes industry

D. Lorente, S. Duarte Serna, E. Betoret, N. Betoret

CAPÍTULO DE LIBRO

Lorente, D., Duarte, S., Betoret, E., & Betoret, N. (2023). Opportunities for the valorization of waste generated by the plant-based milk substitutes industry. Editor(s): Angelo Basile, Alfredo Cassano, Carmela Conidi. In *Advanced Technologies in Wastewater Treatment* (pp. 25-66). Elsevier. https://doi.org/10.1016/B978-0-323-88510-2.00004-X

1. INTRODUCCIÓN

1.1. Opportunities for the valorization of waste generated by the plant-based milk substitutes industry.

Health concerns are increasingly present in today's society. That is the reason why consumers are demanding more and more the presence of healthy products on the market; therefore, the development of new products is a priority for companies in order to satisfy the consumers' demand (Butnariu and Sarac, 2019). Some of the many products that are part of this healthy trend are vegetable drinks or also called plant-based beverages (Sethi et al., 2016).

Plant-based beverages are drinks made from a vegetable raw material, mainly cereals but also nuts and legumes. Although their appearance is very reminiscent of milk, their composition and nutritive value are very diverse and, in some countries, they cannot legally be named vegetable milks, as the term "milk" can only be referred to the secretion of the mammary gland of food-producing animals. It is, therefore, not a milk substitute even though the consumption pattern is very similar to that; a large number of consumers turn to this type of beverage to replace milk to some extent, either because they are lactose intolerant, cow's milk allergic or for ideological reasons. It is worth mentioning vegans, people who do not consume any animal products, including meat, milk, eggs, or honey among other products in the same category, represent a very remarkable portion within the consumers of plant-based drinks. They usually turn to this type of product to replace milk. Worldwide, 11% of people claim to be vegetarians and 3% vegans, which involves an increase of 52% and 153% respectively, since 2016 (Silva et al., 2020). It is also of great interest

to those who want to prevent hypercholesterolemia due to the absence of cholesterol in these beverages.

More and more plant-based drinks are becoming available in supermarkets, whereas a few years ago it was a rarer product. The market for plant-based drinks is continuously growing. For instance, in North America, milk consumption per capita has declined over the last decade while there has been a substantial growth in the plant-based milk alternative beverage industry over the last few decades (Chalupa-Krebzdak et al., 2018). In Spain, the consumption of vegetable drinks was 226104.33 L in 2017, while in 2019 it increased to 228565.57 L, evidencing the growth of this trend (MAPAMA, 2019). Soy drink was one of the first to become popular due to its nutritional composition, but nowadays, cereals, nuts and oilseed are on trend due to their functional properties. Some of the raw materials include rice, oats, spelt, quinoa, walnuts, almonds, hazelnuts, canary seed, sesame, coconut, tiger nut, etc. Simultaneously, soybean beverages are losing popularity because they have a lower consumer sensory acceptability (Sethi et al., 2016).

While there is no official classification for plant-based beverages, Sethi et al. (2016) suggest the following five categories as the main groups:

- Cereal based beverages including oat, rice, corn, and spelt wheat drinks.
- Pseudo-cereal-based beverages including quinoa and amaranth drinks.
- Nut based beverages including almond, coconut, hazelnut, pistachio, and walnut drinks.
- Legume based beverages including soy, peanut, lupine and bean drinks.
- Seed based beverages including sesame, linseed, sunflower seed, and hemp drinks.

Plant-based beverages are rich in antioxidant compounds and help to prevent diseases as mentioned before. Moreover, they are cheaper products compared to

conventional milks. However, plant-based beverages have low bioavailability of mineral and vitamins due to some anti-nutrients and a lack of proteins (Aydar et al., 2020). Only legume-based beverages have a high protein amount (Vanga & Raghavan, 2018). Many of the plant-based drinks on the market are normally enriched with some components, such as calcium or vitamins, to make up for deficiencies in these nutrients. Another solution to compensate the lack of nutrients is to mix two or more kind of beverages, for example, rice and coconut (Vanga & Raghavan, 2018).

The manufacturing process for all these beverages is very similar. The raw material is soaked in water and then it is milled. After that, the liquid phase is separated from the solid one and subjected to homogenization and pasteurization operations to stabilize and commercialize the final product (Rincon et al., 2020). The solid phase left is usually called press cake and it is usually discarded or used as animal feed or fertiliser (Bartkiene et al., 2020a). Although most of the waste generated in the vegetable beverage industry is solid, some liquid waste can also be found. Liquid wastes are those generated in previous stages, such as milling or washing. For instance, in the rice industry wastewater is generated during rice milling. This by-product is commonly called rice mill wastewater.

Nowadays, one of the biggest problems in the food industry is the massive generation of waste and by-products. It is estimated that 1/3 of the food produced is lost during the production process (Comunian et al., 2021a). Governments and other authorities strive to reduce the negative impact of food losses and waste. The European Union is working to implement the goals of the United Nations (UN) 2030 Agenda, which include sustainable development. “Action on sustainable food systems and preventing food waste (SDG 12) will be taken through the EU Platform

on Food Losses and Food Waste, to support the UN target of halving per capita global food waste by 2030. Targeted action is planned to facilitate food donation and the safe use of food not suited for human consumption for production of animal feed as well as more effective date marking on food" (*EUR-Lex - 52016DC0739 - EN - EUR-Lex*, n.d.). At the same time, food companies are working to reduce food waste and to contribute to the circular economy; an economic model in which the value of products, materials and resources remains in the economy for as long as possible, thus reducing waste generation. The current economic model based on extract-produce-consume-throw away is no longer sustainable due to the volume of waste generated (EEA, 2016). In order to contribute to zero waste, research is needed into the potential uses of all food waste, and in particular for that produced by vegetable beverages.

Solid by-products or wastes generated in the plant-based beverage industry contain a considerable amount of nutritional compounds that may be useful for functional foods development (Bartkiene et al., 2020a). Bioactive compounds such as vitamins, phenolic compounds, carotenoids, pigments, and polyunsaturated fatty acid make these by-products very valuable for the industry, even though the bioactive compounds are unstable in some industrial processes (Comunian et al., 2021a). Moreover, there are other by-products generated during the industrial process, especially in the first stages, like the remove of bran layers in cereals or blanching nut skins. These kinds of by-products, that usually are discharged also have a good nutritional profile and therefore, valuable applications in the food industry (Comunian et al., 2021a). Depending on their nutritional composition and properties, they will be more optimal for one use or application. Finally, as a last resort, if the by-product cannot be used directly for the development of a product, the components of interest can be extracted in order not to waste this source of nutrients.

In this context, the aim of this work is to overview the nature, composition and properties of by-products and wastes from plant-based beverage industry and to explore valorisation opportunities in food industry.

1.1.1. Nature of by-products obtained from cereals, nuts, and legumes. Research evolution.

There is very little liquid waste from this industry since the liquid extracted is the intended for sale and consumption. Therefore, liquid wastes are those generated in previous stages, such as milling or washing. The cleaning water can be usually reused with minimum or no treatment as the water condition is relatively pure with the low level of impurities. Contact water, such as soaking and cooking water, is characterised by a high chemical oxygen demand and biochemical oxygen demand due to protein, polypeptide, oligosaccharide and other organic components.

Although most plant-based beverages derive from cereals and nuts, soybean has been crucial in the development of this industry. This legume was the first used to produce a plant-based beverage. It was first consumed in China, and during the second half of the 20th century its consumption spread all over the world (Mordor Intelligence, 2017).

The main solid residue from soybean industrialization is okara. Okara is the by-product obtained from the production of soymilk and tofu. It is the insoluble part of soybeans and looks like an off-white pulp (Harthan & Cherney, 2017). About 1.1 kilogram of fresh okara is produced from each kilogram of soybean processed into soymilk or tofu (Li et al., 2013; Lu et al., 2013a). This by-product is also known as soybean curd residue or soybean residue (Villanueva-Suárez et al., 2013). Most of the okara is dumped, and only on some occasions, it is destined for animal feeding. Due

to the increasing popularity of soybean products during the last decade, okara is the by-product for which more information is available compared to other wastes from plant-based beverage industry. It is considered a very interesting by-product whose nutritional composition and potential uses will be detailed in the following sections.

Rice (*Oryza sativa*) is a monocotyledon crop belonging to the Poaceae family. Worldwide, the annual production of this grain is around 480 million metric tons, growing in more than 100 countries, being China and India the main productors (Amagliani et al., 2017a). It is considered one of the cheapest cereals in terms of production. Therefore, it is predictable that from this amount of rice, a very large quantity of wastes will be generated per year. During the process of rice industrialization, a fraction of the rice grains is usually separated because they are broken. Broken rice makes up 15% of the total processed rice. It is sold at 30–50% of the rice value and usually used for beverages industry or animal feed (da Silva et al., 2020). Besides broken rice, a considerable amount of the grain's outer layers is removed during rice processing, increasing the concentration of nutrients in the bran, and rendering it as an important source of nutrients that seems relevant for the food industry and therefore for human consumption (Faria et al., 2012). Moreover, during rice milling three kinds of by-products are generated: rice mill wastewater, rice husk and rice bran; husk and bran are equivalent to the 20–25% and 10% of the unpeeled grain weight, respectively (Rodríguez-Restrepo et al., 2020a). Rice bran is the major by-product obtained in rice processing (Saman et al., 2019). Bran constitutes approximately 8–10% (w/w) of the whole rice kernel (Sohail et al., 2017). It includes pericarp, seed coat, nucellus, aleurone, pulverized embryo, and some starchy endosperm and hull fragments (Amagliani et al., 2017a). Rice husk is the outer layer of the whole grain, and it is not as useful in the food industry as bran. This is mainly because rice husks are not edible, and although they do have nutrients

of interest, such as antioxidative phenolic compounds, they are difficult to extract, therefore rice husk is less investigated than rice bran (Wanyo et al., 2014). Recently, the valorization of rice bran is gaining importance.

Oat (*Avena sativa*) is a cereal belonging to the Poaceae family. It is an annual crop used for both animal and human nutrition. As it occurs with rice, the main by-product of oat is bran. Oat bran is a very common by-product generated during milling, which constitutes about 50% of the whole grain (based on dry matter content). Specifically, oat bran contains β-glucans, which are proven to have cholesterol-lowering effects (Whitehead et al., 2014). For example, it has been added to meat products to reduce its cholesterol adverse effects (Talukder & Sharma, 2010). Furthermore, some studies have indicated that consumption of oat and barley rich foods may reduce the risk of some chronic diseases such as coronary heart disease, type II diabetes and cancer (Gangopadhyay et al., 2015). Apart from bran, there exist other by-products related to oat. Starch is the major component of oat kernels and may account up to 60% of the dry weight. As a by-product of oat processing and fractionation, the starch can also be utilized for food and non-food applications (Zhu, 2017). Oat hull is the outer layer of the entire cereals, being a 20-30% of the total weight of oat. Hulls can be processed to obtain oat hull fibre, which contains a 90% of insoluble fibre (Daou & Zhang, 2012). It is not as widely used in the food industry as bran.

Almond (*Prunus dulcis*) is the nut coming from the almond tree. Covering the kernel or edible part, it has a brown skin, followed by a shell (hardened endocarp), and in the outer layer there is a hull (flesh but thin mesocarp, green shell cover). During almond processing, these layers are removed, reducing the edible part of the almond to a 40%. The main industrial processes of skin obtention are blanching or roasting, and during blanching two other by-products are algo generated: blanched

skin (the layer next to the kernel) and blanch water. Almond hulls are the most discarded by-product annually, with a total of 6 million tonnes (Prgomet et al., 2017). Almond shells are an under-utilized agriculture by-product (Gong et al., 2011). Around 0.8–1.7 M tonnes of almond shells are discarded annually (Prgomet et al., 2017). Although it is not edible, it has numerous applications in the food industry. The main use is the extraction of relevant compounds, like xylitol or xylooligosaccharides (Gong et al., 2011). Another valuable residue is almond press cake. It is the by-product resulting from the pressing of almonds for oil obtention (De Souza et al., 2020). Almond cake, like almond shell, is used to extract valuable components such as oil or proteins. There are many published research works aimed to analyze the effect of extraction methods on the quality and quantity of extracted compounds. However, scientific literature about composition and uses of press cake from almond milk is scarce. Nowadays, there is no term for describing the solid that remains after obtaining an almond beverage. The term "almond pulp" is sometimes used in homemade recipes; however, it is not mentioned in any scientific article. This demonstrates that this by-product has not been studied yet and could be very interesting to research.

The last residue considered in this review comes from tiger nuts. Tiger nut (*Cyperus esculentus*) or “chufa” is a weed plant from tropical and Mediterranean regions. These tubers are mainly used to prepare a milk-like beverage known as “horchata de chufa”, a very popular beverage in the Valencian Community (Sánchez-Zapata et al., 2012a). 50 million L of this drink are consumed every year, generating an estimated value of 60 million euros. During its production, there result different by-products, which can represent up to 60% of the raw material (Roselló-Soto et al., 2019). Food industries elaborate horchata by the mechanical pressing of tubers, followed by the extraction of the liquid fraction (Codina-Torrella et al., 2017). After

the mechanical extraction, the by-product obtained can be pressed again to separate solid and liquid phases. The liquid extraction is called “horchata drained water”. Meanwhile, the solid extraction is known as “solid tiger nut by-product”. The drained water is therefore a liquid that is not very abundant but still contains nutrients. Sánchez-Zapata et al (2012) state that compounds present in this by-product are beneficial from a technological and a health-related point of view. They also stated that if it is treated, it could be reused to reduce water consumption. The solid by-product is an excellent source of insoluble fibre, which is normally destined to organic mass for combustion, compost, and feed production (Verdú et al., 2017). Solid tiger nut by-product can be used in the development of new products, thus responding to society's demand for potential functional foods. Additionally, these by-products could be a valuable source of oil and other compounds such as macro and micronutrients or even bioactive compounds (Roselló-Soto et al., 2018a).

Figure 1.1 shows the evolution in the number of published scientific works related to by-products from plant-based beverages industry and the main areas of research interest. The key words “raw material press cake” include the published works related to by-products from plant-based beverages industry. The addition of the terms "functional food" or "bioactive compounds" allows to discriminate the interest and the application of the by-product considered. To numerically quantify the evolution in the number of published scientific works, the relative increase (in %) has been calculated considering the difference in the number of items between the two periods: 2011-2015 and 2016-2020, referred to the number of items in the period 2011-2015. Additionally, searches for soybean have been merged with okara to consider the more relevant information about this by-product.

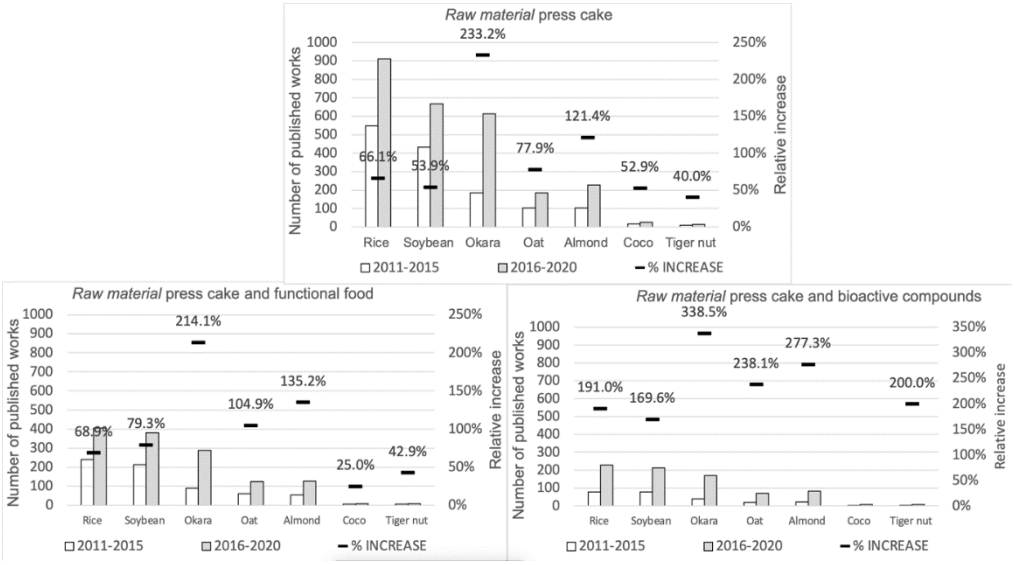

Figure 1.1. Number of scientific published works related to by-products from plant-based beverages industry and functional food area. Relative increase has been calculated as the difference in the number of articles published between the periods 2016-2020 and 2011-2015, divided by the number of articles published in the period 2011-2015, expressed as percentage. The title of each graphic corresponds to the keywords used in the search.

It can be noted in Figure 1.1 that the main by-products considered on scientific research are those from rice and soy, followed by almond and oat. There are a higher number of published works in relation to functional foods than in relation to bioactive components. Although the content of bioactive components is one of the most relevant characteristics that determines the use of these by-products, many studies consider its use as a technological ingredient capable of improving, both the nutritional and the technological properties of the final foods. Regarding the relative increase observed in the last five years compared to the previous five-year period, it is high in all by-products and greater in relation to bioactive compounds. In addition, the number of research works with okara, almonds and oats has increased more than

with the rest of by-products. Although there has been a considerable increase in research in this area, there is still much to investigate. While some wastes have very clear uses, others remain an uncertainty in the food industry.

1.1.2. Nutrient composition.

It is essential to know the composition of considered by-products to define the possible uses and to establish the most important valorization opportunities. By-products composition will determine their nutritional and technological properties. The type and amount of fibre, the lipid profile and the kind and percentage of proteins will determine the nutritive and dietary value, water, and oil interaction properties, foaming capacity, and other relevant properties for food industry applications. Table 1.1 shows published data of macronutrient in the by-products considered in this review.

In addition, it is necessary to consider anti-nutritional factors; those compounds that affect the nutritional value of some foods, especially seeds, as they interfere or inhibit the assimilation of nutrients. Some of these by-products have anti-nutritional factors in their composition. For instance, in rice and oat bran there are polyphenols, oxalates, phytate and trypsin inhibitors. Meanwhile, in soybeans there are saponins, tannins, trypsin inhibitors, oxalate and phytate (Nikmaram et al., 2017). This is the reason why the use of some of these by-products is limited to animal feed (Vong & Liu, 2016). The proper treatment of these compounds can remove these anti-nutritional factors and increase the value of the by-products. The presence of several anti-nutrients in soy compared to other raw materials makes consumers reject soy drinks and choose other alternatives.

INTRODUCCIÓN

Fermentation is one of the best solutions to overcome the presence of anti-nutrients in plant-based beverages. Adeyemo & Onilude (2013) used *Lactobacillus plantarum* and the enzymes produced by this microorganism to reduce the anti-nutritional factors from soybean. Other methods to improve the nutritional quality of legumes and cereals are thermal treatment, enzyme application, soaking, sprouting or irradiation (Nikmaram et al., 2017).

Table 1.1. Macronutrient composition of the different by-products.

BY-PRODUCT	PROTEIN	FAT	DIETARY FIBER	FIBRE SOLUBLE	FIBRE INSOLUBLE	CARBOHYDRATE	UNITS	REFERENCE
By-product from tofu	15.31 ± 0.33	5.90 ± 0.01	58.60 ± 0.20	1.91 ± 0.06	55.63 ± 0.07	n.r	% dry matter	(Lu et al., 2013a)
By-product from tofu	28.52	9.84	55.48	4.71	50.77	2.56	% dry matter	(Redondo-Cuenca et al., 2008)
By-product okara	33.4 ± 0.3	8.5 ± 0.3	54.3 ± 2.3	4.2 ± 1.8	50.1 ± 2.9	3.9 ± 0.2	g/100 g dry matter	(Mateos-Aparicio, Redondo-Cuenca, Villanueva-Suárez, et al., 2010)
Okara (fresh matter)	7.91 ± 0.25	6.22 ± 0.45	13.83 ± 0.49	3.25 ± 0.09	10.58 ± 0.40	2.44 ± 1.29	g/100 g fresh matter	(Guimarães et al., 2018)
Okara	15.2 – 33.4	8.3 – 10.9	42.4 – 58.1	4.2 – 14.6	40.2 – 50.8	3.8 – 5.3	g/100 g dry matter	(Vong & Liu, 2016)
Enzymatic.Hidrolyzed okara	33.4 ± 1.2	9.6 ± 0.1	46.4 ± 2.9	10.7 ± 3.3	35.7 ± 1.3	n.r	g/100 g dry matter	(José Villanueva-Suárez et al., 2013)
Okara	39.1 ± 0.5	16 ± 2	n.r	n.r	n.r	31 ± 5	g/100 g dry matter	(Castellanos Fuentes et al., 2020)
Okara	25.5	12.0	12.2	n.r	n.r	32.6	g/100 g dry matter	(José Villanueva-Suárez et al., 2013)
Rice bran	12.32 ± 0.24	20.31 ± 0.92	28.60 ± 0.32	n.r	n.r	17.92 ± 0.26	g/100 g	(Gul et al., 2015a)
Bran fiber rice	21.91 ± 0.43	4.31 ± 0.43	53.25 ± 0.79	n.r	n.r	1.38 ± 0.18	g/100 g	(Gul et al., 2015a)
Rice bran (Thailand)	11.77 ± 0.0	12.27 ± 0.7	n.r	n.r	n.r	71.04 ± 0.2	% dry matter	(Huang & Lai, 2016)
Rice bran (Korea)	12.32 ± 0.2	20.31 ± 0.9	n.r	n.r	n.r	n.r	% dry matter	(Choi et al., 2011)
Rice bran (Iran)	15.00 ± 0.4	22.40 ± 0.3	n.r	n.r	n.r	n.r	% dry matter	(Rafe et al., 2017)
Rice bran (Portugal)	12.9 ± 0.1	16.53 ± 0.8	n.r	n.r	n.r	62.07 ± 0.2	% dry matter	(Rodríguez-Restrepo et al., 2020b)
Rice bran (Colombia)	12.8 ± 0.3	13.9 ± 0.2	n.r	n.r	n.r	56.2 ± 3.5	% dry matter	(Rodríguez-Restrepo et al., 2020b)
Rice bran	14.7 ± 0.03	20.9 ± 0.20	6.66 ± 0.05	n.r	n.r	52.3 ± 0.26	% w/w	(Amagliani et al., 2017b)
Rice bran (Thailand)	10.90 ± 0.09	12.45 ± 0.23	13.51 ± 2.08	n.r	n.r	45.31 ± 1.00	% dry matter	(Moongngarm et al., 2012)
Rice bran (Thailand)	10.73 ± 0.06	11.62 ± 0.64	10.97 ± 0.07	n.r	n.r	47.56 ± 0.82	% dry matter	(Moongngarm et al., 2012)
Black rice bran (Thailand)	11.73 ± 0.07	11.04 ± 0.14	11.95 ± 0.15	n.r	n.r	47.86 ± 1.12	% dry matter	(Moongngarm et al., 2012)
Red rice bran (Thailand)	10.01 ± 0.6	10.80 ± 0.08	10.16 ± 0.19	n.r	n.r	49.96 ± 1.34	% dry matter	(Moongngarm et al., 2012)
Stabilized rice bran	17.5	13.1	23.34	2.17	21.17	52.33	g/100 g fresh matter	(Bhosale & Vijayalakshmi, 2015)
Probiotic rice bran	19.25	17.2	14.90	1.80	13.10	48.55	g/100 g fresh matter	(Bhosale & Vijayalakshmi, 2015)
Whole rice bran	16.61	17.87	24.15	1.48	22.67	33.24	Dry weight basis	(Faria et al., 2012)
Treated rice bran	19.38	20.05	25.38	0.74	24.64	28.21	Dry weight basis	(Faria et al., 2012)
Roasted rice bran	18.93	18.34	20.45	0.11	20.34	33.76	Dry weight basis	(Faria et al., 2012)
Oat bran	17.93 ± 0.15	6.84 ± 0.02	15.55 ± 1.05	n.r	11.47 ± 0.92	n.r	g/100g dry matter	(Nedeljković et al., 2017)
Oat bran extract	13.1 ± 0.4	<1.7	1.1	n.r	n.r	39.45	% fresh matter	(Ralla et al., 2018)
Oat bran	17.0	7.5	17	n.r	n.r	46	g/100 g	(Herranen et al, 2010)
Oat bran	16.6 ± 0.56	7.5 ± 0.65	16.5 ± 0.84	7.9 ± 0.49	8.5 ± 0.38	n.r	% fresh matter	(Talukder & Sharma, 2010)
Oat hull	n.r	n.r	n.r	n.r	n.r	90	% fresh matter	(Daou & Zhang, 2012)
Press cake almond	7.34 ± 0.01	3.63 ± 0.09	63.68 ± 0.01	n.r	n.r	78.97 ± 0.27	g/ kg fresh matter	(Aydos et al., 2019)
Wheat flour	9.8	1.4	2.3	n.r	n.r	75.1	g/100 g	(Pineli et al., 2015a)
Baru flour (almond)	29.46 ± 1.04	11.84 ± 0.69	38.80 ± 3.74	5.07 ± 1.31	33.73 ± 2.43	11.57	g/100 g	(Pineli et al., 2015a)
Almond press cake	37.20 ± 0.72	16.25 ± 0.79	n.r	n.r	n.r	n.r	% fresh matter	(T. S. P. Souza et al., 2019)
Tiger nut (Egypt)	4.33	22.14	15.47	n.r	n.r	n.r	% dry matter	(Adel et al., 2015)
Solid by-product tiger nut	1.75 ± 0.12	8.85 ± 1.11	59.71 ± 0.03	0.11	59.61 ± 0.08	n.r	g/100 g fresh weigth	(Sánchez-Zapata et al., 2009a)
Horchata drained water	16.90 ± 0.05	8.27 ± 0.03	n.r	n.r	n.r	67.44 ± 0.13	g/100 g fresh weigth	(Sánchez-Zapata et al., 2012b)
Tiger nut milk	1.1	3.9	2.35	n.r	n.r	1.83	% flour weight	(Aguilar et al., 2015)
Tiger nut milk by-product	5.5	16.0	30.55	n.r	n.r	39.6	% flour weight	(Aguilar et al., 2015)
Tiger nut flour	5.1	28.4	19.25	n.r	n.r	38.0	% flour weight	(Aguilar et al., 2015)

1.1.2.1. Okara

Variety of raw material, geographical location or processing factors are some of the reasons why the nutritional values differ among raw materials. Soybean grains usually have variations in their chemical composition depending on the variety, cultural practices, solar incidence, and other environmental factors. Therefore, during the water-soluble soybean extraction process the product characteristics can vary depending on the feedstock used (Guimarães et al., 2018).

In relation to its chemical composition, okara has high moisture content, which results in short durability and difficulty in conservation and commercialization (Guimarães et al., 2018). This high moisture content, which is around 70–80%, makes it susceptible to spoilage and most of it is dumped and burned as waste (Yang et al., 2020). This is the main disadvantage related to the utilization of this by-product. However, some solutions proposed by different authors will be discussed below.

The main carbohydrate in okara is cellulose, which is a very rich source of dietary fibre. The insoluble dietary fibre (IDF) accounts for more than 90% of the total dietary fibre and has the potential to be used in the food industry as a functional ingredient (X. Wang et al., 2020). IDF increases the fecal bulk and reduces the gastrointestinal transit time. Moreover, it seems to have a positive effect on diarrhea and constipation and as a treatment for irritable bowel (Mateos-Aparicio et al., 2010). Other compounds of okara dietary fibre are arabinose, galactose, xylose, and galacturonic acid (Lu et al., 2013b). In addition, okara has a considerable amount of high-quality proteins and especially, essential amino acids. Soybean curd residue contains about 27% of protein (dry basis) with good nutritional quality and a superior protein efficiency ratio, which shows a good potential source of vegetable protein for

human consumption (Villanueva-Suárez et al., 2013). Moreover, its technological properties, such as emulsification, water and fat binding, and foaming properties, make it suitable for its extraction and isolation. The most predominant amino acids in okara are glutamic acid, aspartic acid, and tyrosine *plus* phenylalanine (Li et al., 2013).

Okara contains other components with a notorious importance, such as isoflavones. About 12-30% of the isoflavones in soybeans remain in okara during soymilk production. These isoflavones are mainly glucosides (28.9%), but there are also aglycones (15.4%) and a smaller quantity of acetyl genistin (0.89%) (Vong & Liu, 2016). Research suggests soybean isoflavone affects resistance to cancer, prevents osteoporosis, diminishes antibacterial inflammation, and controls cardiovascular disease (Villanueva-Suárez et al., 2013).

Finally, minerals are not as relevant as the previous components, but they also bring benefits to the by-product, such as antioxidant capacity. The main microelement in okara is potassium, but there is also iron, manganese and zinc, among others.

1.1.2.2. Rice

Carbohydrates represent the major constituents of cereals, including rice. Around 34-62% of carbohydrates are reported to be available in bran (Amagliani et al., 2017a). Starch is the most abundant carbohydrate in rice; however, it is more prevalent in the grain than in the bran. The carbohydrates present in rice bran include arabinoxylans, glucans and hemicellulose (Gul et al., 2015b).

Rice fibre is mainly concentrated in the outer layers of the rice caryopsis (Amagliani et al., 2017a). Few articles detail the percentage of insoluble and soluble dietary fibre, but according to the table, insoluble fibre is quite more abundant in bran than soluble fibre (in particular, it is 16.1 times more abundant).

Proteins present in rice bran are glutelin (22 - 45%), globulin (13 - 36%), albumin (12.5 - 43%) and a few portions of prolamin (1 - 5%) (Rodríguez-Restrepo et al., 2020a). Nutritional quality of rice proteins is estimated to be equivalent or higher than other cereals but considerably lower than proteins derived from animal sources, legumes, and oilseed crops. There are studies on different methods of extraction in order to use rice proteins as value-added ingredients in nutritional products, including sport nutrition supplements and infant formulas (Amagliani et al., 2017a).

Minerals are generally more concentrated in the outer layers of rice grain, being mainly distributed between rice bran (around 72%) and rice endosperm or white rice (around 28%). As it is well known, mineral content is strongly influenced by cultivation conditions, including soil structure condition and fertilization, as well as by rice processing (Faria et al., 2012).

Utilization of rice bran is limited due to enzymatic activity after rice dehulling. This by-product is rich in lipids and an intense lipase activity in the presence of endogenous lipoxygenase causes rapid deterioration of these lipids by rancidification. Because of lipid susceptibility, the commercial use of rice bran requires enzymatic inactivation immediately after bran separation to avoid fatty acid liberation, extend its shelf life and allow its commercialization for human consumption (Faria et al., 2012). Therefore, the method of stabilization results to be very important because it modifies bran composition.

Rice husk is less popular than bran. It contains an antioxidant defense system to protect the rice seed from oxidative stress. The major phenolic compounds present in husk are ferulic acid and p-coumaric acid along with isovitexin, a potent antioxidant (B. Singh, 2018).

1.1.2.3. Oat

Carbohydrates are the major constituent of oat bran, especially starch but also arabinoxylan and oligosaccharides. Besides carbohydrates, oat contains proteins such as globulins and α- and β-polypeptides that account for around 80% of the total protein content, as well as albumins, prolamins, and glutelins. Ralla et al. (2018) demonstrated that oat bran extract containing surface-active saponins and proteins might be used as a natural emulsifier.

One very valuable component in oat is β-glucan. Oat β-glucan is a linear polysaccharide (1→3), (1→4) -β-D-glucan which is a soluble oat fibre being able to attenuate blood postprandial glycemic and insulinemic responses, as well as to lower blood total cholesterol and low-density lipoprotein cholesterol. In addition, it can improve high-density lipoprotein cholesterol and blood lipid profiles as well as to maintain body weight (Daou & Zhang, 2012).

The high fibre content of the oat bran is followed by a good source of B complex vitamins, fat, minerals, tocopherols, and phenolic compounds (Patel, 2015).

1.1.2.4. Almond

Few information about composition of almond by-products is available. The main contribution about macronutrients highlights its fibre as insoluble and an outstanding content compared to other nuts.

The main insoluble fibre-forming compounds in the almond shell are cellulose, ranging from 30 to 50%, hemicellulose in 19-29% and lignin in 20-50% (Prgomet et al., 2017). Most of the published research works seek to determine phenolic compounds profile and antioxidant properties.

Phenolic compounds are aromatic secondary metabolites in plants, which are found largely in fruits, vegetables, cereals, and beverages, and constitute a main part of the human diet.

Some of the most popular phenolic compounds are resveratrol in wine, and isoflavones that are abundant in soya. Almonds are important sources of phenolic compounds. Especially almond hulls, almond skins, almond shells, and almond fruits contain those compounds (Barreira et al., 2010). Naringenin is the most abundant identified compound followed by kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, kaempferol and eriodictyol-7-*O*-glucoside. Blanched skin was found to be the richest in all the classes of compounds analysed and the highest in scavenging and cytoprotective activity (Smeriglio et al., 2016). In particular, it is estimated that around 60-80% of the almond phenolic compounds are found in the skin. However, during the blanching process some of these compounds can be lost because of the exposure of the almond to high temperature. Even though almond skin is exposed to lower temperature compared to roasting (2 min under a temperature of almost 100 °C is the time and temperature used in the industry for blanching), polyphenols are easily lost by hot water blanching (Prgomet et al., 2017).

Almond shell contains trace amounts of phenolics compounds such as caffeic acid, ferulic acid, quercetin, sinapic acid, kaempferol, isorhamnetin and p-coumaric acid. These phenolic compounds have antioxidant activity due to their redox properties.

Some of the antioxidant compounds present in almond hull are chlorogenic acid, cryptochlorogenic acid, and neochlorogenic acid (Meshkini, 2016).

Phenolic compounds are also found in blanched skins and blanching water. Smeriglio et al. (2016) characterized the phenolic content and antioxidant activity of blanched skin and blanch water within the natural almond skin. The results showed that the total phenolic content expressed in mg GAE/100 g of fresh weight was 703.0 ± 15.9 for natural skin, 313.8 ± 2.3 for blanched skin, and 73.9 ± 0.5 for blanch water. Water solubility of these compounds determines their presence or absence. The presence of antioxidant compounds with high added value highlights the potential use of blanch water in the nutraceutical industries (Prgomet et al., 2017).

In particular, blanch water precipitate compounds such as nonpolar aglycones (isorhamnetin and kaempferol), partially insoluble glycosides (quercetin glycoside, kaempferol glycoside, rutinoside, isorhamnetin glycoside, rutinoside, and naringenin glucoside), and even more water-soluble catechins were found. This demonstrates that all these compounds permeate from the skin into blanch water, dissolve to their limit of solubility, and then precipitate out (Prgomet et al., 2017).

1.1.2.5. Tiger nut

Available data reveal tiger nut to be rich in essential dietary constituents such as proteins (3.28 – 8.45%), fats (22.14 – 44.92%), fibres (8.26 – 15.47%) and ashes (1.60 – 2.60%). The industrially relevant recoverable compounds are starch, soluble carbohydrates (mainly in the form of *horchata*), lipids and fibres (Roselló-Soto et al., 2018b). Solid by-product from horchata has an outstanding dietary fibre amount, being the main component of interest in this kind of products. The 99.8% of this dietary fibre is insoluble.

However, it is generally accepted that fibre sources suitable to be used as food ingredients should have a ratio soluble dietary fibre to insoluble dietary fibre close to 1/3. Tiger nut by-product, adequately complemented with other ingredients could be used in dietetic-physiological products. The intake of insoluble dietary fibre is related to the feeling of satiety, as fibre absorbs water, takes up space in the stomach and reduces the need to consume more food (Sánchez-Zapata et al., 2013).

This tuber is also notable for its high fat content, which is the reason why the tiger nut is often a good raw material for oil extraction. In particular, the lipid profile of the tiger nut includes a high percentage of monounsaturated fatty acids, being oleic acid the most abundant of all the monounsaturated fatty acids. In smaller proportion, there are polyunsaturated acids, such as linoleic acid (Roselló-Soto et al., 2019). In fact, there are some research works evaluating the impact of different techniques of oil extraction from tiger nut by-products. For example, Roselló-Soto et al. (2019) studied the effect of supercritical carbon dioxide extraction at different pressures for oil recovering and the results were so positive. The content of α-tocopherol was higher than that obtained with conventional methods of oil extraction. Additionally, it increased the presence of phenolic compounds and showed lower levels of oxidation, avoiding the use of toxic organic solvents usually used in conventional extractions.

Generally, in the extraction processes, the method used determine quality and nutritional value of the final product. Cold press extraction using organic solvents is considered the conventional extraction mode, used for industrial production of tiger nut oil (Roselló-Soto et al., 2019).

Horchata drained water is a valuable source of antioxidants due to the presence of phenols and their reducing power and lipid peroxidation inhibition. In particular,

Sánchez-Zapata et al. (2012) determined that the total phenolic content in horchata drained water was 169.8 ± 10.5 mg GAE/L. These compounds come from the tiger nut cell wall and from food processing, like organic or inorganic cleaning agents (Sánchez-Zapata et al., 2012a).

Some of the phenolic compounds presents in tiger nut are p-hydroxybenzoic acid, vanillic acid, p-hydroxybenzaldehyde, vanillin, p-trans-coumaric acid, trans-ferulic acid, p-cis-coumaric acid or cis-ferulic acid, and they all contribute to improve the antioxidant capacity. Besides that, in tiger nut there are presence of tocopherols, which are described as the most remarkable natural group of antioxidants present in vegetable oils (Gasparre et al., 2020).

1.1.3. Applications in food industry.

In order to establish the applications at industrial level, it is crucial to know the main technological properties of solid waste and the pre-treatments to reduce the biological oxygen demand in wastewater. The following subsections explore these issues in more detail.

1.1.4. Solid wastes or by-products.

The composition and properties of the different by-products are essential to determine their potential uses. The way in which different compounds interact in a food is also conditioned by their chemical structure and the food structure. Interactions among components (micro and macronutrients including water), the length and branching of polysaccharide chains, the solubility of proteins, and the level of unsaturation of fats are examples of some of the characteristics that will

determine functionality of by-products and the most appropriate applications in each case (Figure 1.2).

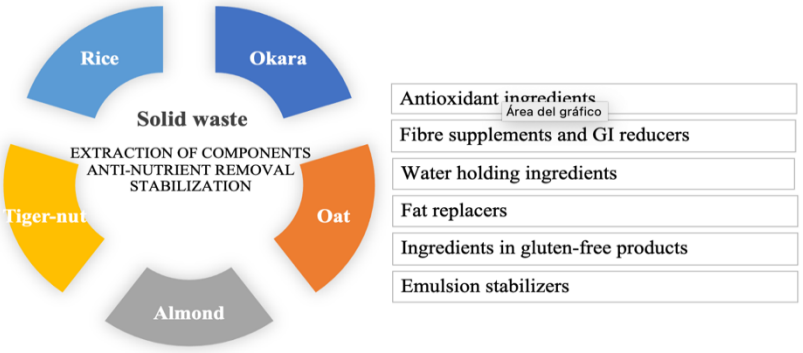

Figure 1.2. Main solid waste origin and applications.

Table 1.2 summarizes the main applications of by-products from plant-based beverages industry considered in the published research works from 2011 to 2020. The most common applications derive from their fibre and antioxidant content and include their use as antioxidants, fibre supplements, fat replacers, gluten replacers, emulsion stabilizers and water holding ingredients. The specific relations among components, properties and uses are discussed below.

The use of by-products as a substrate for the growth of some microorganisms is a very useful application. However, there is not much information on this. Albuquerque et al. (2016) supplemented starter and probiotic strains adding okara in the growth media to increase the folate production, achieving positive results. Castellanos Fuentes et al. (2020) also proved that okara is an adequate substrate for the production of functional ingredients containing probiotic microorganisms. Therefore, it is a relevant application.

Table 1.2. Potential uses of the different by-products.

BY-PRODUCT	FEATURED PROPERTY	POTENTIAL USE	REFERENCE
Okaro	Rich in dietary fiber	Substrate for preparing food ingredients containing *L. casei* ATCC 393	(Castellanos Fuentes et al., 2020)
Fermented okara	Anti-osteoporotic effects	Products with high anti-osteoporotic effects	(Yang et al., 2020)
Fermented okara	Good water and oil holding capacity and swelling capacity index	Technological ingredient in food industry	(X. Wang et al., 2020)
Fermented okara	Higher water absorption capacity and emulsifying properties	Functional and healthy food products	(Y. Hu et al., 2019)
Okara	High fiber content	Formulation of enriched vegetal paste	(Guimarães et al., 2018)
Okara protein isolate	Low solubility	Additive for different food systems	(Fierens et al., 2016)
Okara powder	Rich in nutritional and functional compounds	Substrate to folate production by starter and probiotic cultures	(Albuquerque et al., 2016)
Enzymatically hydrolyzed okara byproduct	Water and oil retention capacity, swelling capacity.	Extraction of polysaccharides, functional ingredients, substrate in *in vitro* fermentability.	(Villanueva-Suárez et al., 2013)
Okara powder	Low glycemic index	Flour replacer to make noodles, bread for diabetics	(Lu et al., 2013)
Soybean curd residue	Swelling and water retention capacity	Texturing additive	(Mateos-Aparicio et al., 2010)
Okara treated with high hydrostatic pressure.	Higher oil retention capacity	Extraction of soluble dietary fiber	(Mateos-Aparicio et al., 2010)
Fermented rice bran and fermented rice flour	Antioxidant properties	Gluten-free cookies	(Christ-Ribeiro et al., 2021)
Water-soluble extract of rice by-product	-	Lactose-free ice cream	(Da Silva et al., 2020)
Rice flour with konjac flour	Increased viscosity, breakdown, and hot paste viscosity	Gluten-free bakery products	(Xu et al., 2020)
Rice flour	Hardness and chewiness	Gluten-free rice bread	(Wu et al., 2019)
Rice bran and broken rice	-	Lactose-free drinks	(Costa et al., 2017)
Rice flour, starch and protein	Dough consistency, high hydration capacity	Gluten-free cookies	(Mancebo et al., 2016).
Rice flour with transglutaminase	Water and oil absorption capacity	Gluten-free cookies	(Altindag et al., 2015)
Stabilized rice bran or its components	Water and fat absorption, emulsifying and foaming capacity	Supplements in various food matrices (bread, pizza, tuna oil, milk powder…)	(Gul et al., 2015)
Rice flour	High viscosity	Gluten-free rice bread	(Alvarez-Jubete et al., 2010)
Rice bran oil	Fat stabilizer	Production of fried snacks, beef patties, etc.	(Lerma-García et al., 2009)
Oat bran	Gelling capacity	To maintain firmness and avoid syneresis in petit suisse cheese	(Lima Ribeiro et al., 2021)
Oat bran	Gelling capacity	Partial fat replacer in cookies	(Milićević et al., 2020)
Oat protein	Faster onset of gel formation	Reduction of syneresis in yogurt	(Brückner-Gühmann et al., 2019)
Oat bran	Emulsifying properties	To stabilize emulsions, prevent coalescence	(Huc-Mathis et al., 2019)
Oat bran	Gelling capacity	Partial fat replacer	(Nedeljković et al., 2017)
Oat starch	Swelling capacity	Fat replacer	(Zhu, 2017)
Oat bran	Lowering glycemic impact	Fibre-enriched extruded snacks	(Brennan et al., 2012)

Oat bran	Water holding capacity and emulsion stability	Fiber enricher in chicken patties	(Talukder & Sharma, 2010)
Oat starch and protein	Foaming capacity and emulsifying activity	To improve cake properties	(Mirmoghtadaie et al., 2009)
Almond press cake from oil extraction	Foaming capacity	Substrate for production of protein and lipids	(De Souza et al., 2020)
Partially delipidified almond flour	-	Cookies elaboration	(Barreira et al., 2019)
Blanched almond skins	Water binding capacity	Composite dough with wheat flour	(Pasqualone et al., 2018)
Almond hulls	High antioxidant capacity	Protection against oxidative damage and membrane protein degradation	(Meshkini, 2016)
Flour (byproduct from oil extraction)	Antioxidant capacity	Cookie elaboration	(Pineli et al., 2015)
Almond cake	Antioxidant capacity	Natural antioxidant	(Iia Sarkis et al., n.d.)
Almond husk	Lipid peroxidation inhibition	Natural antioxidant	(Barreira et al., 2010)
Tiger nut flour	Limits starch degradation during extrusion and increases antioxidant activity	Gluten free snacks	(Gasparre et al., 2020)
Tiger nut flour	High emulsifying ability	Gluten free bakery	(Roselló-Soto et al., 2018)
Tiger nut fiber	Extraordinary water and oil holding capacities	Healthier fiber-rich meat products	(Roselló-Soto et al., 2018)
Unsaturated fatty acids of tiger nut fiber	Beneficial fatty acid profile	To improve nutritional quality of Longaniza de Pascua	(Roselló-Soto et al., 2018)
Tiger nuts liquid by-products	Antioxidant capacity	Water replacer on meat	(Roselló-Soto et al., 2018)
Tiger nut wet fibrous flour	Low wettability and diffusion of water and oil	To produce bread, cakes, and snacks	(Verdú et al., 2017)
Tiger nut flour	Emulsifying and shortening capacity	Gluten free bakery	(Aguilar et al., 2015)
Solid tiger nut byproduct	-	Gluten free bakery	(Aguilar et al., 2015)
Horchata drained water	Natural antioxidant	Substitute of water addition to foods	(Sánchez-Zapata et al., 2012)
Horchata drained water	Important antioxidant properties	Inhibition of lipid peroxidation	(Sánchez-Zapata et al., 2012)
Dietary fiber powder	Low water absorption and adsorption, high emulsifying activity and stability	Fibre enricher in food products	(Sánchez-Zapata et al., 2009)
Dietary fiber powder	High water holding capacity	Products requiring hydration, viscosity development, and freshness preservation	(Sánchez-Zapata et al., 2009)
Solid tiger nut byproduct	High oil-holding capacity	Cooked meat products	(Sánchez-Zapata et al., 2009)
Solid tiger nut byproduct	High emulsifying activity and great emulsion stability	-	(Sánchez-Zapata et al., 2009)

1.1.5. Antioxidants.

Antioxidants are substances found in certain foods that act to protect the body from the action of free radicals which cause ageing processes and some other diseases. The human body cannot neutralize free radicals, so it is through the diet that it obtains antioxidants (also named antiradicals) with the capacity to neutralize them. When antioxidants are not available, oxidative damage such as lipid peroxidation, DNA degradation, protein modification, and inflammation occur, worsening human health. Epidemiological studies have demonstrated that following a diet rich in antioxidants prevents oxidative stress associated diseases due to the inhibition of macromolecules oxidation (Meshkini, 2016).

The best-known antioxidant substances are vitamins C, E, and carotenoids. However, there are other compounds with this feature, such as polyphenols, including flavonoids (flavones, isoflavones, flavonones, anthocyanins and catechins) which are strong antioxidants and contribute significantly to the total antioxidant capacity. Plant products are the main source of antioxidants in diet. Moreover, compounds with antioxidant capacity are usually concentrated in the by-products generated after plant products processing, even more than in the edible part. Concerning cereals, nuts and pulses used to obtain vegetable beverages, different studies provide the total antioxidant capacity as well as the identification of specific components in the by-products. The most relevant ones are highlighted below.

Processed foods rich in fats need the addition of antioxidants to prevent fat oxidation. By-products from almond or the drain water from horchata can perform this function efficiently. For instance, Sánchez-Zapata et al. (2012) demonstrates how the level of inhibition of lipid oxidation is directly proportional to the concentration of horchata drained-water, making it a very profitable natural antioxidant for the

industry. Almond husk, almond cake and blanched almond skin also showed a good antioxidant capacity according to various authors, as they have a high content of phenolic compounds, being suitable for the elaboration of functional foods (Barreira et al., 2010.; lia Sarkis et al., 2014; Pasqualone et al., 2018).

Rice bran is an excellent source of antioxidant compounds, such as polyphenols like gallic acid, protocathecoic acid and coumaric acid, tocopherols, tocotrienols and gamma-oryzanol, which can prevent the oxidation reaction of cells that cause tissue and DNA damage (Christ-Ribeiro et al., 2021).

1.1.6. Fibre supplement and glycemic index reducer.

The glycemic index (GI) indicates the time it takes for a food to raise the blood glucose level. A high glycemic index means a food releases glucose very quickly when eaten. Using glucose as the reference (GI=100), a GI ≤55 is considered low, a GI between 56–69 is considered medium, and a GI ≥70 is considered high.

Dietary fibre lowers the glycemic index by delaying the release of glucose into the blood. There are several mechanisms by which glucose absorption in intestine is slowed down by fibre, such as viscosity increase of intestinal juice, which impedes the glucose diffusion, or the glucose binding which decreases the available glucose concentration in the intestine (Lu et al., 2013b). Another mechanism is the retard of α-amylase action through capsuling starch granules, delaying its degradation and therefore glucose release (Brennan et al., 2012). Insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) have different effects on blood glucose lowering. SDF can delay gastric emptying and form membranes in gastric and intestine, thereby slowing the digestion and absorption of food. IDF absorbs glucose to retard its absorption by intestine (Lu et al., 2013b). Another well-studied effect of fibre is its ability to lower

the level of cholesterol in blood through different mechanisms, but all of them based on avoiding the absorption of cholesterol. Some of the dietary fibre substances are pectins, cellulose, β-glucans, or hydrocolloids (Mudgil & Barak, 2013). From a dietary point of view, it is desirable that the ratio of soluble fibre to insoluble fibre in a food should be around 2/1 (Bas-Bellver et al., 2020). This should be considered when using any of the by-products as an ingredient to increase the fibre content.

As mentioned before, almost all of the dietary fibre from okara is insoluble, therefore its incorporation into functional foods can lower the glycemic index. For instance, Lu et al. (2013) incorporated okara powder into bread, noodles, and steamed bread and the glucose levels after eating were lower than that of control foods (same products but elaborated only with conventional ingredients, without okara). These products are very suitable for diabetic population.

The inclusion of oat bran into snack products prolonged the glucose release period and demonstrated potential to boost satiety. Oat bran can contribute to lowering the glycemic impact of foods through the effect of β-glucans contained in oat bran altering the rheological nature of food digesta. The mechanism of glycemic response delay is the starch encapsulation (Brennan et al., 2012). β-glucans have also a cholesterol lowering ability due to their capacity to form a gel-like network modifying the regular gastrointestinal viscosity and impeding the absorption of cholesterol (Mudgil & Barak, 2013).

High fibre content in rice bran can also slow down the absorption of glucose, being very suitable for the treatment of type 2 diabetes mellitus. Supplementation of rice bran has been successfully carried in various foods like bread, cakes, noodles, pasta, and ice creams without significantly affecting the functional and textural

properties. Therefore, addition of rice bran can contribute to the development of functional foods, which are highly demanded nowadays (Gul et al., 2015c).

1.1.7. Water holding ingredients.

Water holding capacity (WHC) is defined as the amount of water retained by a certain quantity of dry fibres under specified conditions (Mudgil & Barak, 2013). In meat products, this is a very relevant value since a high water-holding capacity avoids exudation phenomena and determines the visual acceptability, weight loss, and cook yield as well as sensory traits on consumption (Warner, 2017).

Water-holding capacity is mainly determined by dietary fibre. Talukder & Sharma (2010) added oat bran to chicken meat patties in order to increase the fibre content, and they showed that the more oat bran added, the higher the WHC. WHC also depends on the way that fibre is processed and on its chemical and physical structure. Tiger nut solid by-product presents a high WHC due to the high proportion of hemicellulose and lignin and the structure of fibre, becoming suitable when added to products that need hydration or viscosity development, such as baked foods or cooked meat products (Sánchez-Zapata et al., 2009b).

1.1.8. Fat replacers.

Fat replacers are either fat substitutes (lipid-like substances) or mimetics (protein or carbohydrate substances). The largest group of fat replacers are carbohydrate-based ingredients, which function by imitating the functional and sensory properties of real fats. In addition to the ability to mimic fat, some replacers possess functional constituents such as fibres. This can improve the functional profile of final products. Cereal milling fractions are used to replace fat, in particular corn bran fibre in muffins,

rye or rice bran in meatballs or wheat and oat bran fibres in dry fermented sausages. They are by-products with confirmed fat replacing abilities (Milićević et al., 2020).

Significant decline in cholesterol content was observed on addition of wheat and oat bran to patties (Talukder & Sharma, 2010). Milićević et al. (2020) revealed that fat replacement using bran gels at the level of 30% resulted in the fat-reduced added-value cookies in terms of dietary fibre, minerals and phenolics.

1.1.9. Ingredients in gluten-free products.

Gluten is a set of proteins present in cereals such as wheat, barley or spelt. It is composed by glutenin and gliadin, and they are responsible for the elasticity of the flour dough and gives the consistency and sponginess of breads and baked doughs. There are disorders related to gluten intake, celiac disease being the best known of them. The prevalence of celiac disease among the general population is estimated to be 1% in Western nations, and there is growing evidence for underdiagnosis of the disease, especially in non-Western nations (Green et al., 2015).

Interest in developing gluten-free products has been growing over the last few years. The development of these kind of products is challenging because gluten confers unique viscoelastic properties to dough, making it difficult to elaborate gluten-free bread formulations with high quality and a competitive price (Aguilar et al., 2015).

Most rice by-products, such as rice bran, rice husk or broken rice, are milled into flour. In fact, rice flour is the mostly used gluten-free flour for bakery products, and it is often formulated with flours, starches, and proteins from cereals, pulses, pseudo cereals, and other plant materials to achieve optimal batter or dough properties and

bakery product quality (Xu et al., 2020). Different milling methods have been reported to affect the properties of rice flour significantly, especially the particle size of the rice flour, the content of damaged starch, and the state of the starch granules (T. Wu et al., 2019). This flour is commonly used for gluten-free bread due to its rheological properties, but it can be used for the elaboration of cookies or other bakery products, depending on the formulation. Additionally, baru (Brazilian almond) flour from oil extraction has been used as a replacer of wheat flour for cookie production, having a positive influence on nutritional and antioxidant characteristics of cookies, besides the impact on acceptance (Pineli et al., 2015b).

Unlike cereals, almonds are not starchy, so their flours do not present functional properties of those polysaccharides in dough. Starch in grain flours fills gluten network, absorbs water during cooking, and as main structural effect it can be observed a tenderization of dough and setting of structure during baking. Together with water, starch makes up more than half the volume of dough. The hydrolysis of starch, also contributes to the availability of fermentable sugars, influencing the production of gas and aeration of dough. However, the reduction of carbohydrates caused by the use of partially defatted baru flour can contribute to the reduction of glycemic loads, which are currently high in bakery. Moreover, the high fibre content also contributes to this reduction (Pineli et al., 2015b).

Several authors studied the benefits and properties of using tiger nut flour in gluten free bakeries. Tiger nut milk by-product impaired bread quality (darkest colour and hardest crumb) due to its fibre size and content. Consequently, it is preferable to combine tiger nut flour with other flours. For example, the use of tiger nut flour together with chickpea flour could substitute shortening and emulsifier agents without negatively affecting crumb softness (Aguilar et al., 2015). Gasparre et al.

(2020) incorporated 10% of tiger nut flour into rice-based formulation showing that it was suitable for making gluten-free snacks with acceptable physical properties and an increase in ash, protein, and total phenol content. Aguilar et al. (2015) formulated a gluten-free dough with corn starch and tiger nut milk by-product and analysed their properties. The batter showed a solid elastic behaviour and a low bake loss due to the high water-holding capacity of the solid by-product.

1.1.10. Emulsion stabilizers.

An emulsion is a homogeneous mixture of two immiscible liquids, where one is the continuous phase, and the other is dispersed in it. There are emulsions consisting of an oil phase in an aqueous phase, known as oil-in-water emulsions, e.g. milk. There are also water-in-oil emulsions, such as butter. Huc-Mathis et al. (2019) studied the emulsifying properties of oat bran. Oat bran samples were dried and micronized to obtain an oat bran powder. They evaluated if oat bran powder could stabilize oil in water emulsions. The mechanisms of stabilization of the oil droplets were given by insoluble and soluble components. Insoluble fibres maintained the stability through pickering mechanism. Pickering emulsion uses single solid particles as stabilizers, which accumulate at the interface between two immiscible liquids and stabilize the droplets against coalescence (Yang et al., 2017). Besides, soluble components like proteins or pectins stabilize the smallest droplets. Emulsifying properties of oat protein isolate can decrease with succinylation (Mirmoghtadaie et al., 2009).

As mentioned before, tiger nut flour can maintain baking characteristics of bread when it is combined with chickpea flour, replacing emulsifier compounds (Aguilar et al., 2015). Sánchez-Zapata et al. (2009) compared the emulsifying activity and emulsion stability of tiger nut solid by-product with other fibre sources such as chia, passion fruit or lima bean. The emulsifying activity of the tiger nut solid by-product

was 70.33 mL/100 mL, and its emulsion stability was 100 mL/100 mL, being the most efficient compared with the other fibre sources.

Fermented okara with *Kluyveromyces marxianus* shows a wide range of emulsification activity index and emulsification stability, enabling its use as a raw material. Moreover, the fermentation improved the mobility of free water and prevented oil droplet polymerization (Y. Hu et al., 2019).

1.1.11. Liquid wastes.

The protection of the planet has introduced the need for increased sustainability at industrial level. This challenge requires the treatment and reuse of wastewater generated by the vegetable beverage industry. Industries need to reach the zero-waste level, for which they require the implementation of innovative technological solutions based on recycling and reuse. These are the guidelines set by a circular economy that replaces the concept of lifecycle and promotes reduction, alternative reuse, recycling, and recovery of materials in the production/distribution and consumption processes. (Gurreri et al., 2020a).

Liquid wastes generated by the plant- based beverage industry can be used for different purposes. The most studied applications are to reuse in the same process, to produce biohydrogen, for nutrients or bioactive compounds extraction and to use as ingredients (Figure 1.3). The main aspects concerning them are explained below. In all cases it is necessary to carry out pretreatments or alternative treatments, some of which are summarized in Table 1.3.

Table 1.3. Some examples of applications and required treatments for wastewater from plant-based beverage industry.

APPLICATIONS	REQUIRED TREATMENTS	RAW MATERIALS	REFERENCE
Wastewater with the best quality	Ultrafiltration	Wastewater from isolated soy protein production	(Cassini et al., 2010)
Wastewater with the best quality	Electrocoagulation	Wastewater from almond industry	(Valero et al., 2011)
Wastewater with the best quality	Electrodialysis	Wastewater from almond industry	(Valero et al., 2015)
Biohydrogen production	Dark fermentation and mixotrophic microalgae cultivation	Rice mill wastewater	(Liu et al., 2013)
Biohydrogen production	Fermentation by Enterobacter aerogenes and Citrobacter ferundii assisted enzymatic hydrolysis	Rice mill wastewater	(Hassan et al., 2021)
Biohydrogen production	Fermentation by Enterobacter aerogenes and Citrobacter ferundii assisted enzymatic hydrolysis	Rice mill wastewater	(Ramprakash and Muthukumar, 2014)
Biohydrogen production	Fermentation by Enterobacter aerogenes	Rice mill wastewater	(Ramprakash and Muthukumar, 2014
Biohydrogen production	Fermentation by Enterobacter and Clostridium species	Rice mill wastewater	(Ramu et al., 2021)
Recovery of proteins	Ultrafiltration	Soy wastewater	(Cassini et al., 2010)
Recovery of proteins	Ultrafiltration	Soybean wastewater	(Chen et al., 2019)
Recovery of proteins	Dried by using a Mini B-290 lab spray dryer	Soy whey wastewater	(Li et al., 2014)
Recovery of proteins	Ultrafiltration	Rice starch wastewater	(Ngoc Thuc Trinh and Quoc Dat, 2021)
Extraction from Polyhydroxyalkanoate	spray-dried to produce a powder and used from Recombinant Strain of Pseudomonas	Soybean wastewater	(Hokamura et al., 2017)
Recovery of β-glucan	Ultrafiltration	Oat mill waste	(Patsioura et al., 2011)
Protein, oligosaccharide and isoflavone isolation	-	Soy wastewater	(Wang et al., 2019)
Water substitute in different foods	-	Horchata drained water	(Sánchez-Zapata et al., 2012)
Use in liver pate formulation	-	Wastewater from tiger nut	(Sánchez-Zapata et al., 2013)

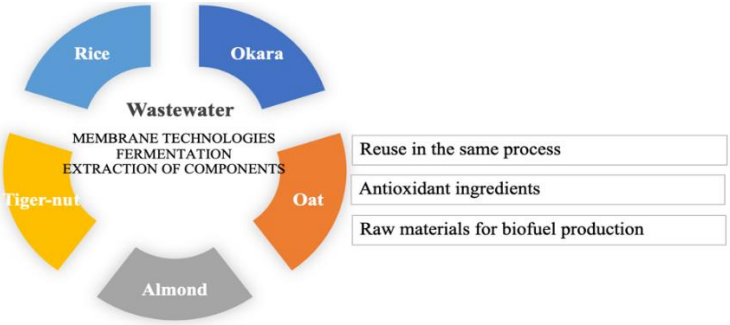

Figure 1.3. Main wastewater treatments and applications and applications.

1.1.12. Reuse in the same process.

Plant based food industry generates large volumes of wastewater containing high concentrations of suspended solids, chemical oxygen demand, conductivity, turbidity or colour among others. Food wastewater cannot be discharged directly and could be reused in the process following treatments that will result in large productive and financial advantages. Traditional physicochemical and biological treatments applied to food liquid wastes demand significant physical space, due to the great volumes of the unit operations involved and implies great chemical product utilization. Thus, alternative treatments have been explored to improve the space economy and significantly reduce the use of chemical product utilization. In this context, membrane technologies have attracted a great deal of research interest and relevant advances have been made. Membrane technologies can be an economically and technologically viable technique for water and wastewater treatment due to high selectivity, high levels of product recovery and avoided use of chemicals (Guerri et al., 2020b). In the treatment of liquid waste of plant origin, two membrane techniques should be highlighted: electrodialysis and ultrafiltration.

Electrodialysis is an electro membrane process that separates ion thanks to the transport through selective membranes under an applied electric field, thus

producing two streams at different concentration (Gurreri et al., 2020a). In almond industry, electrodialysis has been proved to be a suitable technique for decreasing the conductivity of a real wastewater, which was previously treated by electrocoagulation and electrooxidation (Valero et al., 2015). A study of the reuse of the concentrate solution was made, and it was stated that it can be concentrated 10 times. The treatment was successfully scaled-up to a pre-industrial electrodialysis system (Valero et al., 2015).

Ultrafiltration is a membrane process that promotes the separation of molecules in solution based on size, where a pressure gradient is the driving force of the process (Cassini et al., 2010). Common compounds retained by ultrafiltration membranes include colloidal particles, biomolecules, polymers, and some sugars. The production of proteins from some legumes generates wastewater with a very high organic load, composed mainly of soluble proteins and carbohydrates and that have a high chemical oxygen demand. Cassini et al., (2010) studied the use of ultrafiltration to reduce organic load and protein content. They evaluated the performance of three ultrafiltration membranes (5, 20 and 50kDa) in the treatment of wastewater from isolated soy proteins production. The 5kDa membrane presented the best results: the lowest permeate flux reduction, the most elevated retention percentages (34% of chemical oxygen demand, 52% of protein, 21% of total solids and 86% of total suspended solids) and the wastewater with the best final quality.

1.1.13. Biofuel

Hydrogen is considered a biofuel because it uses water to obtain electricity, being considered a clean energy (Liu et al., 2013). Biohydrogen is seen as one of the most viable energy substitutes due to its higher energy content and environmentally friendly nature (Hassan et al., 2021). Biological production or production involving

fermentation processes is one of the most efficient methods as it consumes less energy and can be carried out at atmospheric pressure (Kotay and Das, 2008). It uses agricultural industrial waste rich in starch and cellulosic materials, which are first hydrolysed to produce fermentable sugars (Ramprakash and Muthukumar, 2014). Biological species used for hydrogen production include cyanobacteria, photosynthetic bacteria, and bacterial species, such as facultative anaerobes (Enterobacter sp. and Citrobacter sp.) and strict anaerobes (Clostridium sp. and rumen bacteria) (Das and Veziroğlu, 2001; Kotay and Das, 2008). Bacillus Some industrial wastewaters, such as dairy process wastewater, rice winery wastewater, distillery and molasses wastewater, cassava wastewater and rice mill wastewater, have been successfully used for biohydrogen production (Ramprakash and Muthukumar, 2014) (Ramu et al., 2021) (Ramu et al., 2021). Among the pretreatments used are remarkable the acid, enzymatic and microbial hydrolysis (Ramprakash and Muthukumar, 2014) although others such as heat, ultrasonication and alkali have been also used (Ramprakash and Muthukumar, 2014).

1.1.14. Nutrient extraction

The recovery of nutrients from cereals and legumes wastewater is drawing high interest from researchers. It contributes to circular economy by valorising wastes and reducing the environmental impact. Some applications of proteins or bioactive compounds recovered include the use as food ingredients, algae culture, animal feed and active packaging design (Chen et al., 2019). Membrane separation processes are a sustainable alternative for this recovery, as they greatly reduce energy consumption and the use of chemical agents. Ultrafiltration is a common technique used to recover colloidal particles, biomolecules or polymers although, sometimes, the high viscosity of the wastewater will cause serious membrane fouling and

inefficient separations. For this reason, it is necessary to investigate each specific application and find solutions that provide higher yields.

Proteins from rice are of great interest due to their colourless and bland taste. They are highly digestible protein of high biological value, rich in amino acids such as cysteine, methionine, tryptophan and phenylalanine (Ngoc Thuc Trinh and Quoc Dat, 2021). Thus, the wastewater discharged from rice starch production contains a significant amount of valuable proteins. Ultrafiltration, used by other authors for the recovery of protein from other wastewaters (Hernández et al., 2019; Sriniworn et al., 2016) proved to be an effective method to recover rice protein from wastewater in rice starch production.

Soy protein isolates are a group of macromolecules with different structures and molecular weights, identified as globulins. They have been attributed functional properties such as cholesterol reduction and prevention of cardiovascular diseases, which has increased their interest for the food industry. Wastewater from soy protein isolates production is rich in soluble proteins; small molecules that were not removed during the production process. Cassini et al. (2010) demonstrated that the use of ultrafiltration membranes in the pre-treatment of soy protein isolates wastewater is very promising for organic load reduction and protein content recuperation. Isoflavones are also present in the soy protein isolates wastewater. Although research on the recovery of soy isoflavones is scarce, Xu et al. (2004) demonstrated the efficiency of reverse osmosis to obtain concentrates of these components from the wastewater resulting from the production of soy milk.

Oats are among the cereals that have high amounts of water-soluble fibre, protein and β-glucan. The acceptance of β-glucan from cereals as a functional ingredient has increased the interest for its incorporation in food formulation. Patsioura et al. (2011)

carry out an optimization of ultrafiltration for the recovery of β-glucan from solid and liquid waste from oat mills, using three types of membranes. Although the results were satisfactory, good separation yields between β-glucan and proteins were not obtained.

1.1.15. Direct use as ingredient

When the wastewater does not come from cleaning but from pre-treatments applied to the raw material or from post-treatments of the solid waste, it can be interesting to use the water directly as an ingredient in other food processes.

Processing of tiger nut to obtain horchata generates a second extraction the horchata drained water. Sánchez-Zapata et al. (2012) studied the properties of this wastewater, so that its properties make it a valuable source of natural antioxidants and fibre. The same authors studied the effect on the physicochemical and sensory properties of a cooked pork liver pate when 50% of the water required was replaced by drained horchata water. The results showed no significant changes in the physicochemical properties and revealed a better overall acceptance by a panel of inexperienced tasters.

1.1.16. Conclusions and future trends

After an exhaustive review of scientific published works related to the by-products generated in the production of plant-based beverages and other products, it has been shown that the most investigated by-products are those not derived directly from the production of the vegetable beverages but those that are originated in previous stages (hulls, skins, etc.). Regarding press cakes, there is still not enough information, but some studies carried out show that they have a high potential, both

for the extraction of compounds of interest such as oils, dietary fibre, and antioxidants for the elaboration of functional foods due to their beneficial properties. For wastewater, a large amount of research work focuses on the application of pre-treatments for the reduction of organic load. An improvement in the efficiency of these steps would enable different applications such as the production of biofuels or the recovery of nutrients.

The use of this type of by-products contributes to the circular economy and economically benefits the companies. The plant-based beverage sector is growing, so knowing the best uses for each type of by-product generated in this area is crucial. In fact, the growing number of scientific articles published on these by-products shows that their properties are beginning to be studied, being the relative increase of most articles very significant.

Throughout the work, it has been proved that by-products from plant-based beverages industry have both an interesting nutritional composition and a wide range of applications in the development of new products, besides the fortification of traditional foods. Meat products, emulsions, gluten-free products, dairy products, and others are just some of the many sectors in which these by-products play an important role. This is therefore an area for further research in order to optimise food production processes.

Future research in this field is very promising due to the need to increase the sustainability of the agri-food system by adding value to potentially highly versatile by-products. The development of extraction techniques and the application of innovative technologies as pre-treatment will allow the recovery of many components of interest with multiple applications.

ACRONYMS

GAE: Galic acid equivalents.

GI: Glycaemic index.

IDF: Insoluble dietary fibre.

SDF: Soluble dietary fibre.

WHC: Water holding capacity.

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1.2. Procesos tecnológicos para la transformación y aprovechamiento de residuos y subproductos. Problemas asociados con la obtención y vida útil de productos en polvo.

Los residuos y subproductos de la industria alimentaria pueden utilizarse tanto en la producción de alimentos para animales, como para la obtención de ingredientes y alimentos funcionales de interés para la industria (Bedoić et al., 2019; Comunian et al., 2021b). Tal y como se ha visto en el apartado anterior, muchos de estos subproductos y residuos contienen una cantidad considerable de agua y compuestos bioactivos tales como compuestos fenólicos, carotenoides, ácidos grasos polinsaturados, vitaminas y pigmentos que los hacen altamente inestables y perecederos. Su procesado tecnológico para la obtención de productos en polvo permite aumentar la vida útil ya que contribuye a la reducción de los procesos de oxidación y del crecimiento microbiológico. Los productos en polvo resultantes tienen numerosas aplicaciones sobre todo en productos animales, productos lácteos, bebidas y productos de panadería (Gómez-García et al., 2021).

La obtención de ingredientes o productos en polvos generalmente involucra dos fases fundamentales: la eliminación de agua que puede realizarse por diferentes métodos de deshidratación y el triturado del producto seco para la obtención del polvo con una determinada granulometría (Santos et al., 2022). La deshidratación permite disminuir la actividad del agua y prolongar la vida útil, a la vez que permite concentrar y conservar los compuestos fitoquímicos (Majerska et al., 2019; Sagar et al., 2018; Sepúlveda et al., 2021). Esta operación implica la transferencia de calor y materia produciendo cambios físicos, químicos y transiciones de fase que determinarán las propiedades del producto final y su calidad (Ramírez-Pulido et al.,

2021). A continuación, se explican de forma muy sucinta las operaciones de deshidratación más utilizadas en la industria alimentaria.

1.2.1. Secado por aire caliente (HAD).

El secado por aire caliente o secado convencional es la operación de deshidratación más habitualmente utilizada en la industria alimentaria debido a su sencillez y a sus menores costes de inversión inicial y de operación. En esta operación, la transferencia de calor desde el aire hasta las capas más internas del material a secar ocurre acoplada con el transporte y evaporación de agua hasta alcanzar un nivel seguro, evitando así problemas microbiológicos durante el almacenamiento y transporte (Betoret et al., 2015). En un principio, se elimina el agua libre o no ligada, y a medida que avanza el proceso de secado parte del agua atrapada dentro de la muestra se desplaza hacia la superficie y luego se transfiere a la corriente de aire circundante. Por lo general, la evaporación del agua no ligada ocurre durante lo que se conoce como el período de velocidad de secado constante, mientras que el agua ligada se elimina a lo largo del período de velocidad de secado decreciente (Calín-Sánchez et al., 2020).

Las altas temperaturas empleadas en la mayoría de los procesos de secado por aire caliente (entre 45 y 80 °C) causan daños irreversibles en las estructuras celulares pared y membrana y en las propiedades del producto que determinan su funcionalidad. Estos cambios incluyen la permeabilidad de la membrana y la resistencia mecánica del conjunto pared-membrana. Además, y sobre todo como consecuencia de reacciones de oxidación, las estructuras químicas responsables del valor biológico y de la funcionalidad de los componentes también se ven alteradas. Así, disminuye el valor funcional de compuestos como vitaminas y antioxidantes.

Todos estos cambios afectan la calidad y el valor nutricional del producto deshidratado (Betoret et al., 2015; Korus, 2011). Sin embargo, existen estudios que demuestran que determinadas temperaturas pueden aumentar la capacidad antioxidante. Resultados publicados por Papoutsis et al. (2017) proporcionaron una cantidad superior de ácido gálico en residuos de limón secados a 110 °C. También Que et al. (2008) reportaron un incremento considerable de la capacidad antioxidante en harina de calabaza secada por aire caliente a 70 °C. Bajo estas premisas, el control de las condiciones del proceso resulta fundamental para asegurar la calidad del producto final.

1.2.2. Liofilización (LIO).

La liofilización consiste en la deshidratación mediante la sublimación del agua previamente congelada (Barbosa et al., 2015). Debido a la ausencia de agua en estado líquido y a las bajas temperaturas aplicadas durante el proceso, se detienen la mayoría de los procesos de deterioro y las reacciones microbiológicas, lo que resulta en un producto final de excelente calidad (Broeckx et al., 2016). Las circunstancias bajo las cuales se realiza la liofilización evitan los daños generados por la oxidación durante el proceso de deshidratación. Estas condiciones posibilitan la conservación de una gran cantidad de compuestos volátiles y otros compuestos solubles y fácilmente oxidables, reduciendo al mínimo las alteraciones en la composición química del alimento. Es bien sabido que la liofilización es el mejor método de secado para conservar los compuestos bioactivos u otros componentes nutricionales de los alimentos. Krokida et al. (2000) estudiaron el efecto sobre compuestos volátiles de muestras de manzana durante el secado al aire y la liofilización, y encontraron que la retención del aroma se vio afectada por la temperatura de secado y el método de secado utilizado. Por otra parte, Silva-

Espinoza et al. (2019) reportaron resultados favorables en la conservación de vitamina C, fenoles totales y β-caroteno en puré de naranja liofilizado. Wang et al. (2007) estudió la eficiencia de encapsulación del aroma de los polvos de banano producidos mediante secado en banda, liofilización y secado al vacío, y demostró que los polvos de banano liofilizados tenían un aroma óptimo.

Sin embargo, la etapa de sublimación asociada al proceso de liofilización trae consigo la rotura de las estructuras celulares imposibilitando la rehidratación del producto final. Como consecuencia los productos finales suelen tener una mayor porosidad (Calín-Sánchez et al., 2020). Si se emplea para la producción de productos en polvo suelen obtenerse polvos de menor tamaño de partícula que en muchos casos resultan ser más higroscópicos (Harnkarnsujarit & Charoenrein, 2011; Wei et al., 2022). En algunos casos como los polvos obtenidos por liofilización de arándano y manzana, la higroscopicidad tiende a ser mucho mayor apareciendo problemas de pegajosidad y apelmazamiento (Teng et al., 2020; Wei et al., 2022).

A pesar de sus numerosas ventajas, conviene resaltar que su aplicación a nivel industrial resulta limitada ya que es el proceso más caro para la producción de alimentos deshidratados (Cassani et al., 2020).

1.2.3. Apelmazamiento y vida útil de alimentos en polvo .

El manejo de productos en polvo a granel es una actividad industrial ampliamente practicada en la industria alimentaria. Se busca que los productos fluyan sin restricciones, por lo que las propiedades deben adaptarse a los procesos de fabricación y en las aplicaciones finales (Freeman et al., 2015). No obstante, ciertos materiales tienden a agregar sus partículas formando conglomerados que dificultan

su manejo y disminuyen su calidad. A este fenómeno se le conoce como "apelmazamiento" (Irani et al., 1959; Zhang et al., 2022) y tiene una gran importancia económica (Aguilera et al., 1995). Existen múltiples factores como la temperatura, humedad, velocidad de deformación y vibración que tienen un gran impacto en el fenómeno de apelmazamiento (Zafar et al., 2017).

Los acondicionadores de flujo o agentes antiaglomerantes son aditivos que proporcionan un flujo constante de cualquier masa en polvo (Irani et al., 1959). Los agentes antiaglomerantes tienen una gran importancia en todas las industrias que manejan productos en polvo, especialmente en las industrias farmacéutica, química y alimentaria (Chang et al., 2018; Hollenbach et al., 1982). A menudo, se agregan a los sistemas alimentarios en polvo para mejorar las propiedades físicas y la estabilidad. Los antiaglomerantes o antiapelmazantes pueden ser de síntesis o naturales, orgánicos o inorgánicos. Los silicatos, polisacáridos, fosfatos, estearatos y sales de hierro son los antiaglomerantes inorgánicos más comunes. La mayoría de ellos son insolubles o parcialmente solubles en agua y etanol. Una de las características más importantes de los agentes antiaglomerantes es tener un área superficial alta, de forma que puedan adsorber cantidades significativas de agua (Yapıcı et al., 2021). En los últimos años, se han realizado esfuerzos significativos dirigidos a la obtención de aditivos alimentarios de carácter innovador, con el fin de prevenir el apelmazamiento en los productos en polvo. No obstante, en este contexto, han surgido cuestiones de importancia atribuibles al empleo de aditivos de naturaleza inorgánica en los productos en polvo. Es importante afrontar cuestiones relevantes tales como la seguridad alimentaria, la disminución de los perfiles sensoriales, incluyendo sabor y textura, así como desafíos inherentes a la correcta identificación y transparencia en la información dirigida a los consumidores. Adicionalmente, se han constatado reacciones alérgicas vinculadas a diversos

aditivos; problemas microbiológicos derivados de la continua aplicación de agentes antiaglomerantes; y se ha evidenciado un potencial impacto ambiental derivado de los procesos de manufactura de dichos aditivos. Por esta razón, la utilización de agentes antiaglomerantes naturales tales como la bentonita, el carbono biológico, residuos de aceites vegetales y animales se ha visto en aumento en los últimos años (P. Hu et al., 2014).

Los alimentos en polvo son sensibles al calor, la humedad, el oxígeno y la luz, siendo estos los factores externos más críticos para determinar su vida útil. La susceptibilidad de cada producto a estos factores varía según su composición química y el proceso de fabricación utilizado. El aumento de la actividad de agua tras la exposición a la humedad puede conducir al colapso de la estructura, aumentando la tasa de deterioro y causando cambios físicos y reacciones químicas. Durante el almacenamiento, la accesibilidad de la unión del agua es fundamental para la estabilidad del producto, ya que afecta la velocidad de los cambios físicos más perjudiciales, como la aparición de pegajosidad y apelmazamiento. Estos cambios físicos están condicionados en gran medida por las propiedades de interacción con las moléculas de agua tales como la higroscopicidad, la capacidad de absorción de agua y la de retener agua.

Otra de las causas más comunes del deterioro de los productos en polvo ricos en grasa durante el almacenamiento es la oxidación de lípidos (Barden & Decker, 2016). Tanto las operaciones de procesado como el almacenamiento prolongado aumentan el grado de oxidación de los lípidos (Ahmed et al., 2016). La oxidación de lípidos es un proceso complejo favorecido fundamentalmente por la presencia de oxígeno y las altas temperaturas (Domínguez et al., 2019). Como consecuencia se originan compuestos químicos de diferente naturaleza que afectan en gran medida las

características sensoriales del producto final. Los peróxidos son compuestos de oxidación primaria, y los aldehídos, cetonas, epóxidos, compuestos hidroxilo, oligómeros y polímeros son compuestos de oxidación secundaria, que se usan comúnmente para determinar el grado de oxidación de los lípidos (Kunyaboon et al., 2021). El índice de peróxidos se utiliza en alimentos para determinar su nivel de enranciamiento y deterioro (Hori et al., 2019). Según el real decreto 308/1983 del BOE que aprueba la reglamentación técnico-sanitario de aceites vegetales comestibles, el índice de peróxidos para aceites vegetales tendrá que ser menor a 20 meq de O_2 activo/Kg de grasa.

1.3. La funcionalidad de los alimentos y los modelos de digestión gastrointestinal *in vitro*.

El efecto nutritivo y saludable de los alimentos viene determinado por su contenido en macro y micronutrientes, su liberación a lo largo del tracto gastrointestinal y su asimilación. Estos tres aspectos considerados conjuntamente definen la funcionalidad de un alimento y se reflejan por separado en los conceptos de digestibilidad (*digestibility*), bioaccesibilidad (*bioaccesibility*) y biodisponibilidad (*bioavailability*). La bioaccesibilidad del nutriente se define como la cantidad del nutriente que se libera de la matriz alimentaria y queda disponible para su absorción a través de la pared intestinal. La biodisponibilidad del nutriente considera la cantidad total del nutriente que se libera y absorbe para llegar al torrente sanguíneo desde donde se distribuye a los diferentes tejidos corporales. Además de estos, la digestibilidad se aplica específicamente a la fracción de los componentes de los alimentos que se transforman en materia potencialmente accesible a través de todos los procesos físicos y químicos que tienen lugar en el lumen. Además, recientemente, la influencia de los nutrientes en las rutas metabólicas a través de metabolitos

secundarios producidos por la microbiota intestinal se ha confirmado como decisiva en su efecto saludable (Hadadi et al., 2021).

La bioaccesibilidad suele ser el paso limitante que determina la biodisponibilidad de muchas sustancias bioactivas (Tan et al., 2020). Los avances rápidos en los métodos de digestión *in vitro* están ayudando a esclarecer la relación entre el procesado de los alimentos, su composición y la bioaccesibilidad de sus nutrientes. De hecho, la complejidad estructural de un alimento y cómo se transforma a lo largo del tracto gastrointestinal juegan un papel fundamental en sus propiedades nutritivas y funcionales (H. Singh et al., 2015). El conocimiento detallado de las transformaciones estructurales que tienen lugar en todas las etapas de la cadena de suministro de alimentos y durante la digestión, podría ser utilizado para desarrollar alimentos con mejores propiedades nutritivas y funcionales (Duijsens et al., 2022).

Los modelos de digestión de alimentos *in vitro* están evolucionando hacia modelos más (semi)dinámicos. Los métodos estandarizados y estáticos son herramientas simples, fáciles de manejar y adecuados para obtener resultados preliminares de digestibilidad y bioaccesibilidad. Sin embargo, la digestión es un proceso dinámico donde los alimentos que ingresan al tracto gastrointestinal se transfieren de un compartimento a otro a velocidades variables dependiendo de su estructura, contenido calórico, osmolaridad y propiedades reológicas. Las condiciones fisicoquímicas (pH, fuerza iónica, concentraciones de enzimas digestivas, entre otras) que se dan en los diferentes compartimentos evolucionarán con el tiempo. La obtención de resultados relevantes sobre el efecto nutricional y funcional de un alimento requieren de sistemas dinámicos que se aproximen en mayor medida a la complejidad de los sistemas fisiológicos.

INTRODUCCIÓN

La digestión *in vitro* estática comúnmente utilizada se base en el protocolo internacional INFOGEST Cost Action (Minekus et al., 2014). Se estableció con el objetivo de consolidar las condiciones para la digestión simulada de alimentos de una forma consensuada. Se describió un marco de parámetros para la digestión oral, gástrica y del intestino delgado. De acuerdo con este modelo, para cada etapa de la digestión es necesario la preparación de los fluidos oral (FFS), gástrico (FGS) e intestinal (FIS). En la etapa oral se mezclan 5 g de muestra con 5 mL de FSS que contiene 75 U/mL de amilasa, se ajusta el pH a 7 con NaOH 1M y se incuban en una cámara termostatada a 37 °C durante 2 min. En la etapa gástrica se añaden 10 mL de FGS que contiene 2000 U/mL de pepsina al bolo alimenticio resultante de la fase oral, se ajusta el pH a 3 con HCL 1M y se incuban en una cámara termostatada a 37 °C durante 2 h a 55 rpm. Para la etapa intestinal se añaden 20 mL de FIS que contiene 100 U/mL de pancreatina y 10 Mm de sales biliares al quimo resultante de la fase gástrica, se ajusta el pH a 7 con NaOH 1 M y se incuban en una cámara termostatada a 37 °C durante 2 h a 55 rpm.

Por el contrario, varios modelos multicompartimentales dinámicos se han desarrollado durante las últimas décadas y se han revisado recientemente (Guerra et al., 2012). Uno de los más conocidos es el modelo dinámico multicompartimental SHIME ® que fue desarrollado en la Universidad de Ghent (Bélgica), que representa el tracto gastrointestinal (TGI) del ser humano adulto como lo describe Molly et al. (1993). Este consiste en una sucesión de cinco reactores (estómago, intestino delgado, colon ascendente, transverso y descendente) simulando las diferentes partes del tracto gastrointestinal.

1.3.1. La digestión gastrointestinal y la fermentación colónica.

La digestión de los alimentos implica una serie compleja de procesos físicos y químicos. Estos procesos comprenden la ingesta de alimentos, su descomposición, la absorción de nutrientes esenciales, el transporte a órganos relacionados y la eliminación de desechos. Este proceso se lleva a cabo en el tracto gastrointestinal y requiere la participación de otros órganos auxiliares como las glándulas salivales, el hígado, la vesícula biliar y el páncreas. Cada uno de estos órganos auxiliares desempeña una función específica y en conjunto, extraen los nutrientes de los alimentos digeridos y eliminan los materiales no utilizados.

El proceso se inicia con la etapa oral responsable de la masticación y la deglución del alimento. Durante esta etapa, la saliva y los dientes desempeñan un papel fundamental. En el caso de alimentos sólidos, se lleva a cabo una secuencia de masticación con el objetivo de reducir el tamaño de las partículas del alimento, creando así un bolo seguro (Peyron et al., 2011). Por otro lado, cuando se trata de alimentos líquidos, el proceso de digestión en la fase oral es más rápido y resulta especialmente beneficioso si el alimento contiene almidón (Woda et al., 2006).

El estómago es el órgano que interviene en la digestión gástrica y ejecuta un complejo proceso de descomposición física y química de los alimentos en condiciones ácidas (Bornhorst & Singh, 2014). Los alimentos experimentan modificaciones significativas en su estructura, preparándolos para ser digeridos y absorbidos de manera más eficiente en el intestino delgado (Tran Do & Kong, 2018). En el estómago es donde se inicia la digestión de proteínas y lípidos. En estos cambios resulta fundamental la composición de la fase gástrica regulada por estímulos neurales, hormonales, paracrinos, mecánicos y químicos (Engevik et al., 2020). La fase gástrica incluye ácido clorhídrico, enzimas (pepsina y lipasa gástrica), diversos

electrolitos, la mucosa gástrica (recubrimiento del estómago), factor intrínseco (glicoproteina producida por las células parietales que facilitan la absorción de la vitamina B12) y hormonas. El ácido clorhídrico es responsable de la acidez del líquido gástrico, con un pH de 1,5-3,5, y su producción está regulada hormonalmente por las células parietales de la pared del estómago. Entre los electrolitos que componen el líquido gástrico destacan el potasio, cloruro, sodio, fosfato, carbonato, magnesio, calcio, tiocianato, amonio y urea, entre otros (Minekus et al., 2014). Estos compuestos están relacionados con el control del pH y la regulación de la capacidad amortiguadora. La pepsina es una proteasa aspártica que es activa en un entorno ácido, con un rango de pH de 1,5 a 5, y es responsable de la hidrólisis de proteínas gástricas (aproximadamente el 10 al 15% según el tipo de alimento). Debido a su amplia especificidad, la pepsina produce mezclas heterogéneas de péptidos y polipéptidos de diferentes tamaños de polimerización (Goodman, 2010). Por otro lado, la lipasa gástrica es activa a un pH de 4 a 6 y contribuye a aproximadamente entre el 5 y el 30% de la lipólisis (Carriere et al., 1993).

Durante la digestión en el intestino delgado, el quimo, que es la mezcla de alimentos y fluidos intestinales, se transforma en quilo. El quilo atraviesa tres secciones distintas del intestino delgado: el duodeno, el yeyuno y el íleon. El duodeno conecta al intestino delgado con el hígado través de la vesícula biliar y el páncreas, que son de vital importancia ya que se encargan de liberar las enzimas responsables de la digestión (bilis y pancreatina) (Bornhorst & Singh, 2014). En el duodeno ocurre la mayor parte de la digestión de los nutrientes. En el duodeno, las secreciones pancreáticas que contienen enzimas digestivas y carbonato neutralizan el pH (6–7,5) (Guerra et al., 2012). Las sales biliares juegan un papel crucial en la hidrólisis de lípidos del intestino delgado. En el yeyuno y el íleon sigue el proceso de descomposición de los alimentos y se inicia la absorción de los nutrientes. Esta última

se realiza principalmente en el íleon. El intestino delgado se considera el área principal para la absorción de nutrientes ya que tiene una superficie externa de unos 30 m^2 revestida de vellosidades y microvellosidades (Boland, 2016; Jaime-Fonseca et al., 2016). La absorción de nutrientes compite con el tránsito por el intestino, que dura entre 2 y 5 h (Boland, 2016). Por último, los hidratos de carbono más complejos que no se hidrolizan pasan al intestino grueso para ser fermentados por la microbiota colónico.

La fase colónica es la última etapa de la digestión. El colon o intestino grueso está compuesto por 3 partes: colon ascendente, transverso y descendente. Las principales funciones del colon son almacenar y procesar material residual no digerido. No obstante, durante este período también ocurre la mezcla y absorción de agua, sales y algunos de los productos generados. Este proceso es principalmente resultado de la acción fermentativa de una comunidad de microorganismos (microbiota) que aprovechan la materia no digerida, principalmente fibra y algunas proteínas. El ecosistema microbiano está compuesto principalmente por grupos de bacterias, pero también pueden encontrarse algunos hongos (Macfarlane & Macfarlane, 2011). El colon es un ecosistema microbiano complejo en el que la microbiota incluye miles de especies y subespecies de bacterias diferentes.

1.3.2. La microbiota intestinal, su relación con la dieta y con la salud.

El intestino humano alberga un complejo ecosistema de microorganismos conocido como microbiota intestinal. Esta comunidad microbiana, que se encuentra en una estrecha y simbiótica relación con el huésped, ejerce un gran impacto en diversos aspectos de nuestra fisiología. La microbiota intestinal es extremadamente abundante, comprendiendo miembros de los tres dominios de la vida: bacterias,

Eukarya y Archaea. Se estima que puede alcanzar hasta 10^{14} células microbianas (Oxley et al., 2010; Scanlan & Marchesi, 2008).

La microbiota está principalmente compuesta por algunos filos bacterianos, siendo Firmicutes y Bacteroidetes los predominantes, representando más del 90% de los microorganismos residentes (Eckburg et al., 2005). A pesar de esta relativa simplicidad en términos de filos bacterianos, la microbiota intestinal es excepcionalmente diversa a nivel de especies y cepas. Se han detectado miles de especies en el intestino de la población humana, y cada individuo alberga aproximadamente 160 especies prevalentes (Qin et al., 2010). Es importante destacar que la estructura de la microbiota varía considerablemente de un individuo a otro, con poca superposición filogenética entre personas (Qin et al., 2010; Tap et al., 2009). Además, esta composición puede cambiar rápidamente a lo largo del tiempo en un solo individuo debido a factores ambientales y variabilidad genética (Candela et al., 2012; Faith et al., 2013). Estos cambios en la composición de la microbiota pueden ser observados en un período tan corto como 24 horas después de modificar la dieta, y resultan en diseños metabólicos específicos de la microbiota intestinal-huésped, lo que a su vez afecta al estado de salud y enfermedad del huésped (Muegge et al., 2011). Así, el cambio de una dieta basada en productos vegetales a una basada en productos animales provoca cambios significativos en la composición de la comunidad microbiana y en los productos metabólicos finales en tan solo un día (David et al., 2013). Una dieta rica en polisacáridos procedentes de alimentos vegetales favorece a los microorganismos productores de butirato, como *Roseburia, Eubacterium rectale y Faecalibacterium prausnitzii*. Por lo contrario, una dieta basada en alimentos de origen animal resulta en un aumento de microorganismos potencialmente putrefactivos y tolerantes a la bilis, como *Bilophila wadsworthia y Alistipes.* Mientras que la respuesta de la microbiota a corto plazo a

cambios dietéticos muestra ajustes rápidos en la abundancia relativa de diferentes componentes, los patrones dietéticos a largo plazo se han asociado con estados estables y diferenciados en la composición del ecosistema microbiano intestinal (G. D. Wu et al., 2011).

Diferentes componentes de la dieta influyen en el diseño co-metabólico específico entre el microbiota intestinal y el huésped, lo que da lugar a la producción de productos metabólicos específicos que afectan la fisiología del huésped. Por ejemplo, la presencia de polisacáridos complejos en la dieta promueve un metabolismo sacarolítico en el intestino, seleccionando una comunidad diversa de bacterias especializadas en la degradación de polisacáridos. Estos microorganismos degradadores de polisacáridos establecen una red metabólica sintrófica, lo que conduce a la producción de ácidos grasos de cadena corta (SCFA) que benefician la salud del huésped. Por otro lado, la presencia de proteínas en la dieta selecciona un metabolismo proteolítico en el intestino, pero con una comunidad menos diversa de microorganismos especializados en la degradación de proteínas. Esto resulta en la producción de ácidos grasos de cadena corta y ácidos grasos de cadena ramificada (BCFA), así como otros metabolitos como los fenólicos e indólicos, que pueden estar asociados con problemas de salud, al igual que las metilaminas (Moco et al., 2014).

Por otra parte, los avances recientes en la investigación del microbioma humano han ampliado nuestra comprensión de las diversas comunidades microbianas que forman parte del cuerpo humano. Los estudios sobre la microbiota han revelado que las interacciones entre los microorganismos y el huésped no se limitan a un solo órgano, sino que generan una comunicación cruzada entre distintos sistemas del cuerpo. Se ha prestado particular atención a la microbiota intestinal, demostrando su papel crucial en la regulación de la homeostasis en varios órganos, incluyendo el

tracto gastrointestinal a nivel local, y a nivel sistémico, los pulmones y el cerebro. Entre las funciones más destacadas se encuentran el fortalecimiento de la integridad intestinal, la extracción de energía, la protección contra patógenos y la regulación de la respuesta inmunológica del huésped. Así, su estado se asocia estrechamente con enfermedades como enfermedades hepáticas (Lang et al., 2020), diabetes (Kim et al., 2019), enfermedad inflamatoria intestinal (Malham et al., 2019),enfermedades autoinmunes (Zhao et al., 2017), cáncer colorrectal (Coker et al., 2019) y enfermedades del sistema nervioso central (Malan-Muller et al., 2018).

1.3.3. Los ácidos grasos de cadena corta (SCFA) y su relación con la salud.

Las investigaciones más recientes señalan que gran parte de los beneficios que los alimentos ejercen sobre la salud están relacionados con la producción de ácidos grasos de cadena corta por parte de algunas especies bacterianas que integran la microbiota y cuya presencia se ve favorecida por el consumo de determinado tipo de alimentos (Goswami et al., 2018).

En los apartados anteriores hemos visto que el intestino delgado tiene una función altamente especializada en la descomposición, emulsificación y absorción de nutrientes, lo que asegura que la mayoría de los nutrientes sean completamente digeridos. Normalmente, solo una pequeña cantidad de grasa, carbohidratos simples y proteínas llegan al colon sin ser completamente descompuestos y absorbidos. De esta forma los carbohidratos complejos, como las fibras dietéticas, que no son digeribles en el intestino delgado debido a la falta de enzimas necesarias para su descomposición llegan al colon, donde ciertas bacterias residentes los utilizan como fuente de energía, metabolizándolos en diferentes moléculas llamadas ácidos grasos de cadena corta como el acetato, butirato y propionato. Estos son los SCFA

principales, que representan el 90% de los SCFA, los cuales se producen en proporciones casi constantes de 60:25:15 en términos molares.

Los SCFA son los principales metabolitos producidos en el colon por la fermentación bacteriana de fibras dietéticas y del almidón resistente (Y. P. Silva et al., 2020). Sin embargo, aunque la fermentación anaeróbica de las fibras es la mayor fuente de SCFA también se pueden producir acetato, propionato y butirato a partir del metabolismo de los aminoácidos (Louis & Flint, 2017). Los SCFA promueven la salud intestinal mediante una serie de efectos locales, que incluyen el mantenimiento de la integridad de la barrera intestinal, la producción de moco y la protección contra la inflamación. Además, los SCFA también contribuyen a reducir el riesgo de cáncer colorrectal (Lewis et al., 2010; O'Keefe, 2016).

Entre los múltiples beneficios, el butirato es una fuente de energía esencial para las células del colon humano, promoviendo la apoptosis en las células tumorales del colon y regulando la energía. El butirato contribuye al control de la condición anaeróbica en el colon al activar la β-oxidación en las mitocondrias. Por otro lado, el propionato desempeña un papel en la regulación de la gluconeogénesis a nivel hepático. Además del propionato y el butirato, también se está investigando el impacto del succinato. El succinato es más conocido como un intermediario del ciclo de Krebs y se considera un sustrato para la fosforilación oxidativa mitocondrial, pero también es un producto metabólico de las bacterias. En este contexto, el succinato se ha ignorado clásicamente porque se consideraba principalmente como un intermediario clave en la síntesis de propionato. Actualmente, ha habido informes de asociaciones tanto beneficiosas como opuestas entre el succinato y la resistencia a la insulina, la obesidad y la inflamación (De Vadder et al., 2016; Wan et al., 2020).

Por último, el acetato, además de afectar a los tejidos periféricos, es necesario para el crecimiento de bacterias (Valdes et al., 2018).

1.4. Los procesos de fermentación en el aprovechamiento de subproductos o residuos agroalimentarios.

La fermentación es un método tradicional de procesado de alimentos que se viene utilizando desde la antigüedad, sobre todo para conservar los alimentos. Tiene lugar en presencia de microorganismos beneficiosos como levaduras, mohos y bacterias, que descomponen los azúcares y almidones en alcoholes y ácidos, incrementando así el valor nutricional y modificando las propiedades sensoriales de los alimentos (Adebo et al., 2017; Ilango & Antony, 2021). Los microorganismos más comúnmente utilizados en los procesos de fermentación son las bacterias (Sadh et al., 2018)del ácido acético, las bacterias del ácido láctico, las levaduras o los hongos filamentosos (Marco et al., 2021). Aunque tradicionalmente la operación se realizaba de forma espontánea a partir de la comunidad bacteriana autóctona de la materia prima, en los últimos años se ha favorecido la adición de cultivos iniciadores. El uso de una especie microbiológica específica como inóculo puede reducir en gran medida los efectos de los microorganismos de deterioro, inhibir el crecimiento de microorganismos patógenos y controlar el proceso, reduciendo el tiempo necesario y mejorando la calidad sensorial e higiénica del producto final. Además, el uso de iniciadores puede dar lugar a un producto final con un efecto específico sobre la salud e impulsar la selección y el desarrollo de nuevos probióticos.

Además, estudios recientes indican que el consumo habitual de alimentos fermentados puede disminuir el riesgo de padecer enfermedades promoviendo un adecuado estado de salud (Martínez-González et al., 2019). Aunque los mecanismos por los que los alimentos fermentados pueden contribuir a mantener una buena

salud y prevenir numerosas enfermedades siguen sin estar claros, los avances tecnológicos y la investigación están arrojando luz sobre este campo. Algunas de las razones previamente identificadas son que algunos microorganismos pueden mejorar la digestibilidad de los macronutrientes, contribuir a mejorar la diversidad de la microbiota intestinal y reforzar el sistema inmunitario, y producir metabolitos secundarios con un efecto beneficioso. En este contexto, se está llevando a cabo una evaluación científica y técnica relacionada con el uso de microorganismos con efecto probiótico como cultivos iniciadores. Esta evaluación requiere valorar su supervivencia en el tracto gastrointestinal mediante modelos de digestión *in vitro* y avanzar en la aplicación de tecnologías que mejoren su resistencia y viabilidad.

En los últimos años se ha incrementado la investigación relacionada con la fermentación de subproductos o residuos de la industria agroalimentaria. La fermentación en estado sólido, en la que los microorganismos se cultivan en la superficie de un sustrato insoluble en agua (W. Zhang et al., 2015), se presenta como una oportunidad para aprovechar y dar valor a los residuos agroindustriales (Sadh et al., 2018). La riqueza en carbohidratos de mayor o menor complejidad de muchos de los residuos procedentes de cereales, legumbres, frutas y hortalizas los convierten en sustratos idóneos para ser fermentados. En los casos en los que predominan en gran medida compuestos lignocelulósicos de gran resistencia serán necesarios procesos previos de hidrólisis que liberen moléculas más simples y susceptibles de ser utilizadas por los microorganismos (Bhusari et al., 2014; Singh Duhan et al., 2013). Una de las aplicaciones que ha alcanzado un gran desarrollo con resultados prometedores es la obtención de biocombustibles a partir de la fermentación de residuos lignocelulósicos (Najafi et al., 2009).

En el caso de residuos y subproductos con mayor contenido en carbohidratos simples, la fermentación puede aplicarse con la finalidad de realizar un aprovechamiento integral y obtener ingredientes con propiedades tecnológicas y funcionales interesantes. Diferentes estudios ponen de manifiesto la mejora en el perfil nutricional de subproductos de hortalizas cuando se someten a un proceso de fermentación. Entre los beneficios en el perfil nutricional que se asocian con la fermentación de residuos, es importante resaltar el incremento en la capacidad antirradical del producto final (Gulsunoglu et al., 2020). Esto puede deberse tanto a la producción de nuevos compuestos con actividad antioxidante por parte de los microorganismos que realizan la fermentación (Bei et al., 2017), como a la transformación de algunos compuestos antioxidantes en otros con una capacidad antirradical mayor (Bier et al., 2019). También se han observado otros beneficios como el aumento en la digestibilidad o la eliminación de componentes antinutritivos. Adicionalmente, la utilización de microorganismos con efecto probiótico o la producción de metabolitos secundarios por parte de estos podría tener un efecto positivo en la prevención de enfermedades cardiovasculares, el cáncer, el síndrome del intestino irritable, la anorexia nerviosa y la diabetes (Patel et al., 2023; Xiang et al., 2019).

1.4.1. Los microorganismos con efecto probiótico en el aprovechamiento de subproductos y residuos. Estrategias para promover su viabilidad y mecanismos de adaptación.

**(Este apartado se ha desarrollado en mayor medida en el capítulo 3 de la tesis dedicado a la fermentación de bagazo de almendra para la obtención de un producto probiótico).*

Los probióticos se definen como "microorganismos vivos que, cuando se administran en cantidades adecuadas (10^8 -10^9 UFC/día), confieren un efecto beneficioso sobre la salud del huésped" (Hill et al., 2014). De hecho, estos microorganismos han atraído una atención especial debido a sus propiedades funcionales y nutricionales, y se han desarrollado varios estudios para aclarar los mecanismos de acción en el cuerpo humano (Marzorati et al., 2021). A pesar de que la industria de alimentos está trabajando en la creación de nuevos alimentos probióticos diferentes a los lácteos como los zumos, cereales, snacks o suplementos (Aspri et al., 2020; Granato et al., 2010; Rivera-Espinoza & Gallardo-Navarro, 2010), preservar la viabilidad del microorganismo probiótico en un nuevo producto siempre representa un desafío, debido a que diversos factores pueden ocasionar pérdida de viabilidad. Factores como la temperatura de almacenamiento, la humedad, el pH, condiciones de uso, distribución y las condiciones del proceso son responsables de reducir el crecimiento de las bacterias probióticas. A partir de lo mencionado previamente, se destaca la importancia de garantizar la viabilidad y actividad metabólica de los microorganismos probióticos desde la etapa de elaboración de los alimentos hasta su consumo por parte de los consumidores (Almada-Érix et al., 2021; Ejtahed et al., 2012) así como durante su tránsito por el tracto gastrointestinal (Hill et al., 2014; Marzorati et al., 2021). Para que un alimento pueda ser tener alegaciones de salud y sea catalogado como probiótico es necesario que su contenido en microorganismos probióticos sea de al menos 6 log CFU/g, y se recomienda un consumo de al menos 9 log CFU/dia para tener alegaciones de salud no específicas de la cepa probiótica (Bautista-Gallego et al., 2019). En el desarrollo de un alimento funcional probiótico pueden destacarse tres etapas en las que se agrupan los factores externos de estrés que afectan a la viabilidad de los microorganismos:

- **Etapa 1:** Crecimiento del microorganismo y transferencia al alimento.

Incluye el conjunto de operaciones utilizadas para favorecer el crecimiento del organismo que puede realizarse en un medio óptimo de crecimiento o en la materia prima alimentaria que se transformará en el producto final. En esta etapa los factores que limitan la viabilidad de los microorganismos probióticos son los relacionados con la composición nutricional del medio de crecimiento, así como el posible aumento de sustancias inhibidoras de microorganismos como las bacteriocinas.

- **Etapa 2**: Procesado del alimento y almacenamiento hasta su consumo.

Incluye el conjunto de operaciones necesarias para transformar la materia prima alimentaria en el producto final (mezclado, deshidratación, concentración, horneado, cocción, salazón…) así como las condiciones óptimas de almacenamiento del alimento hasta su consumo. En esta etapa los factores que limitan la viabilidad de los microorganismos son la temperatura, la acidez, la presencia de sal o azúcar y la presencia de oxígeno.

- **Etapa 3:** Digestión gastrointestinal.

Durante la digestión los microorganismos probióticos deben tener la capacidad de sobrevivir a las condiciones del estómago y del intestino delgado y a su vez ser capaces de adherirse y colonizar la mucosa intestinal. En última instancia, los microorganismos podrían ejercer sus efectos sobre la salud produciendo compuestos antimicrobianos, compitiendo por sitios de adhesión y nutrientes con otras especies y estabilizando la microflora intestinal.

Debido a la dificultad de alcanzar niveles de 6-9 log CFU/g o ml en los alimentos probióticos sin lácteos al final de la digestión gastrointestinal, se han desarrollado

diferentes estrategias para aumentar la viabilidad de los microorganismos. Estas estrategias pueden clasificarse en dos grandes líneas de investigación. Por una parte, la primera está basada en la **protección** de las cepas por inclusión de las mismas en estructuras protectoras. Por otra parte, la segunda está basada en la exposición de los microorganismos probióticos a condiciones desfavorables, pero no letales para favorecer la aparición de cambios en los mismos que promuevan su **adaptación**.

Entre las estrategias dirigidas a la **protección** de los microorganismos puede destacarse:

- La microencapsulación.

  La encapsulación es un proceso mediante el cual un material o mezcla de materiales se recubre o queda atrapado dentro de otro material o sistema. El recubrimiento protege el contenido activo del estrés ambiental como la acidez, el oxígeno y las condiciones gástricas. Existen diferentes métodos de encapsulación como son el secado por aspersión, el secado por congelación o al vacío, recubrimiento de lecho fluido, enfriamiento por aspersión, encapsulación por emulsificación, entre otros.

- La inclusión de probióticos en películas protectoras o recubrimientos.

Entre las estrategias dirigidas a la adaptación de los microorganismos puede destacarse:

- Fermentación controlada. Adaptación de las condiciones de proceso a la flora autóctona o a los cultivos iniciadores utilizados.

  El avance en las fermentaciones ha impulsado la necesidad de seleccionar microorganismos adecuados para la producción industrial de alimentos. Se han

identificado diversos microorganismos con características específicas para productos alimenticios, como *Saccharomyces cerevisiae* en bebidas alcohólicas, *Lactobacillus* en productos lácteos fermentados y productos cárnicos, así como mohos en la producción de alimentos a base de soja como miso, shoyu y tempeh (Chen et al., 2016; Yulifianti & Ginting, 2018). Estos microorganismos se utilizan como cultivos iniciadores para acelerar la fermentación. La selección de estos cultivos implica un proceso sistemático que incluye la evaluación de características como tolerancia al estrés, la producción de metabolitos clave, su seguridad y otros parámetros tecnológicos. Los cultivos iniciadores son ricos en microorganismos deseados y se utilizan para acelerar la fermentación, pero necesitan adaptarse al entorno para permitir un control óptimo del proceso.

- Selección de especies y evolución dirigida.

La evolución dirigida es un proceso basado en la teoría de la selección natural de Darwin. Implica la adaptación a un entorno muy estresante mediante mutaciones espontáneas del ADN. Los organismos que han adquirido mutaciones ventajosas prosperan, se reproducen y se convierten en dominantes en condiciones de estrés específicas.

- Inducción de un estrés controlado e incorporación posterior.

Las bacterias probióticas han desarrollado mecanismos de protección contra diversos factores estresantes, tanto ambientales como fisiológicos. Estas estrategias de defensa son específicas para cada especie y cepa de bacterias probióticas. Se ha demostrado que los probióticos aumentan su tolerancia al estrés y su viabilidad cuando se exponen a condiciones subletales gradualmente crecientes. (Teixeira et al., 1994, 1995). Además, muchos estudios han demostrado que los probióticos adaptados a múltiples factores pueden

desarrollar tolerancias cruzadas y por tanto aumentar su viabilidad y supervivencia a lo largo del tracto gastrointestinal (Weiss & Jespersen, 2010).

- Utilización de cepas probióticas que formen esporas.

Existe un grupo de bacterias con la capacidad de formar esporas, entre las cuales se destacan aquellas pertenecientes a los géneros *Bacillus, Clostridium, Sporolactobacillus* y *Brevibacillus* (Hong et al., 2005). Las esporas tienen la capacidad de resistir tratamientos extremos que, en circunstancias normales, serían letales para otras bacterias. Estos tratamientos incluyen altas temperaturas, radiaciones ionizantes, disolventes químicos, detergentes y enzimas hidrolíticas (Errington, 2003; Nicholson et al., 2000). Existen múltiples informes que destacan la asombrosa capacidad de las esporas para mantener su viabilidad durante largos períodos de tiempo, posiblemente incluso millones de años (Cano & Borucki, 1995; Errington, 2003; Vreeland et al., 2000). Las esporas de Bacillus tienen una larga trayectoria de uso en humanos y animales. Su consumo se ha demostrado seguro y se está investigado su posible utilización como microorganismo probiótico. (Lee et al. (2012) informaron que un compuesto similar a la surfactina, producido por B. subtilis CSY191, podría inhibir el crecimiento de células cancerosas. Además, la fermentación en estado sólido de Bacillus amyloliquefaciens en soja puede utilizarse para la producción de 1-desoxinojirimicina, un potente inhibidor de la α-glucosidasa (Cai et al., 2017). La pasta de soja fermentada por B. licheniformis 67 ha demostrado tener un efecto positivo en la prevención de la obesidad en un modelo animal, como se informó en el estudio de (Choi et al., 2016).

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2. OBJETIVOS Y PLAN DE TRABAJO

2. OBJETIVOS Y PLAN DE TRABAJO

2.1. Objetivos

El objetivo general de la presente tesis doctoral es estudiar las posibilidades de revalorización del subproducto resultante del proceso de obtención de la bebida vegetal de almendra (en adelante bagazo de almendra). Determinar las propiedades fisicoquímicas, tecnológicas y funcionales de la materia prima; evaluar el efecto de la operación de deshidratación sobre las propiedades del producto deshidratado, el contenido en componentes bioactivos, su bioaccesibilidad y su influencia sobre la microbiota. Finalmente, se consideró la posibilidad de obtener un producto deshidratado con probióticos.

Para el cumplimiento del objetivo general, se plantearon los siguientes objetivos específicos (OE):

OE1. Determinar el efecto de la temperatura de secado por aire caliente, de la liofilización y del triturado posterior sobre las propiedades fisico-químicas, tecnológicas y funcionales, así como sobre la estabilidad durante el almacenamiento de un polvo de elevado valor nutricional obtenido a partir del bagazo de almendra.

OE2. Estudiar la posibilidad de utilizar el subproducto deshidratado y triturado como ingrediente sustitutorio parcial de la harina de trigo en la elaboración de galletas.

OE3. Determinar el efecto de la deshidratación y el triturado sobre el contenido en componentes bioactivos y su bioaccesibilidad durante el proceso de digestión gastrointestinal *in vitro*. Establecer la influencia sobre la microbiota en el proceso de fermentación colónica *in vitro*.

OE4. Determinar el efecto de la fermentación, la incorporación directa y la encapsulación mediante presiones de homogenización sobre la resistencia al proceso de deshidratación, la digestión y la hidrofobicidad de un microorganismo probiótico incorporado al bagazo de almendra.

2.2. Plan de trabajo

Se presenta a continuación el plan de trabajo propuesto para lograr los objetivos establecidos.

2.2.1. Obtención y caracterización del bagazo de almendra deshidratado y triturado.

- Obtención del subproducto de la bebida vegetal de almendra y caracterización en términos de composición nutricional (agua, proteína, fibra, grasa y cenizas), propiedades fisicoquímicas (actividad de agua, contenido en sólidos solubles, tamaño de partícula y color) y propiedades funcionales (contenido en polifenoles y actividad antirradical)
- Deshidratación y triturado del bagazo de almendra. Como métodos de deshidratación se aplicarán el secado por aire caliente a 60 y 70 °C y la liofilización.
- Determinación de la cinética de secado a las temperaturas de 60 y 70 °C. Establecer el tiempo de secado necesario para que el producto final alcance una actividad de agua inferior a 0,3.
- Determinación de la composición nutricional (agua, proteína, fibra, grasa y cenizas), propiedades fisicoquímicas (actividad de agua, contenido en sólidos solubles, tamaño de partícula y color), tecnológicas (propiedades de interacción con el agua y con el aceite) y propiedades funcionales

(contenido en polifenoles y actividad antirradical) del bagazo de almendra deshidratado y triturado en las diferentes condiciones. Obtención de las isotermas de sorción a 20 °C.

- Estudio de la estabilidad durante el almacenamiento normal y acelerado (6 meses) del bagazo de almendra deshidratado y triturado en las diferentes condiciones. Se determinará el índice de peróxidos, la evolución en las principales propiedades fisicoquímicas (actividad de agua, humedad y color), tecnológicas (higroscopicidad, acidez y índice de peróxidos) y funcionales (contenido en polifenoles y actividad antirradical).

La Figura 2.1 muestra en forma de diagrama de flujo las operaciones y las condiciones de proceso para la obtención del polvo de bagazo de almendra, y las determinaciones analíticas previstas.

2.2.2. Aplicación de los diferentes polvos de bagazo de almendra en la formulación de productos de panadería.

- Formulación, preparación y caracterización de mezclas de harina de trigo con bagazo de almendra deshidratado a 60 °C o liofilizado con proporciones de sustitución de la harina de trigo del 10%, 15% y 25%. Determinación de las propiedades fisicoquímicas (actividad de agua, humedad y color), tecnológicas (propiedades de interacción con el agua y con el aceite y propiedades reológicas) y funcionales (contenido en polifenoles y actividad antirradical) de las mezclas.
- Preparación de galletas a partir de las mezclas de harina de trigo con bagazo de almendra deshidratado. Determinación del efecto del

porcentaje de sustitución sobre las propiedades fisicoquímicas y funcionales de las galletas.

La Figura 2.2 muestra el proceso de formulación, preparación y caracterización de las mezclas y de las galletas.

2.2.3. Ensayo de digestión gastrointestinal y fermentación colónica *in vitro* del bagazo de almendra deshidratado y triturado en las diferentes condiciones.

- Determinación del efecto de las etapas oral, gástrica e intestinal sobre las propiedades funcionales (contenido en polifenoles y actividad antirradical).
- Determinación de la influencia del bagazo de almendra deshidratado y triturado en las diferentes condiciones sobre la estructura poblacional de géneros bacterianos con capacidad fermentativa generados durante la fermentación colónica *in vitro* utilizando inóculos de adultos sanos. Identificación de especies con potencial efecto beneficioso en población adulta sana.
- Determinación del perfil de ácidos grasos de cadena corta generados durante el proceso de fermentación colónica *in vitro* del bagazo de almendra seco por aire caliente y liofilizado utilizando inóculos de adultos sanos.

La Figura 2.3 muestra el proceso de digestión gastrointestinal y fermentación colónica *in vitro* del bagazo de almendra deshidratado y triturado en las diferentes condiciones de proceso.

2.2.4. Ensayo de incorporación del probiótico *Lactobacillus salivarius* spp. al bagazo de almendra fresco.

- Inoculación del probiótico *Lactobacillus salivarius* spp. y fermentación (24 h, 37 °C) del bagazo de almendra fresco.
- Crecimiento, separación de células por centrifugación e incorporación del probiótico *Lactobacillus salivarius* spp. en el bagazo de almendra fresco.
- Crecimiento, separación de células por centrifugación, encapsulación mediante presiones de homogenización (70 MPa) e incorporación del probiótico *Lactobacillus salivarius* spp. en el bagazo de almendra fresco.
- Refrigeración durante 24 horas a 4 °C, y posterior secado con aire durante 23 horas a 50 °C de los bagazos de almendra frescos con probiótico.
- Simulación de la digestión gastrointestinal *in vitro* de los bagazos de almendra deshidratados con probiótico.
- Determinación del efecto del método de incorporación del probiótico y de la operación de secado por aire caliente sobre la viabilidad del probiótico y su hidrofobicidad. Valoración de la viabilidad tras el proceso de digestión gastrointestinal *in vitro.*

En la Figura 2.4, se presentan los diversos procesos que involucran la incorporación y la simulación de la digestión *in vitro* del probiótico *Lactobacillus salivarius* spp. en el bagazo de almendra fresco

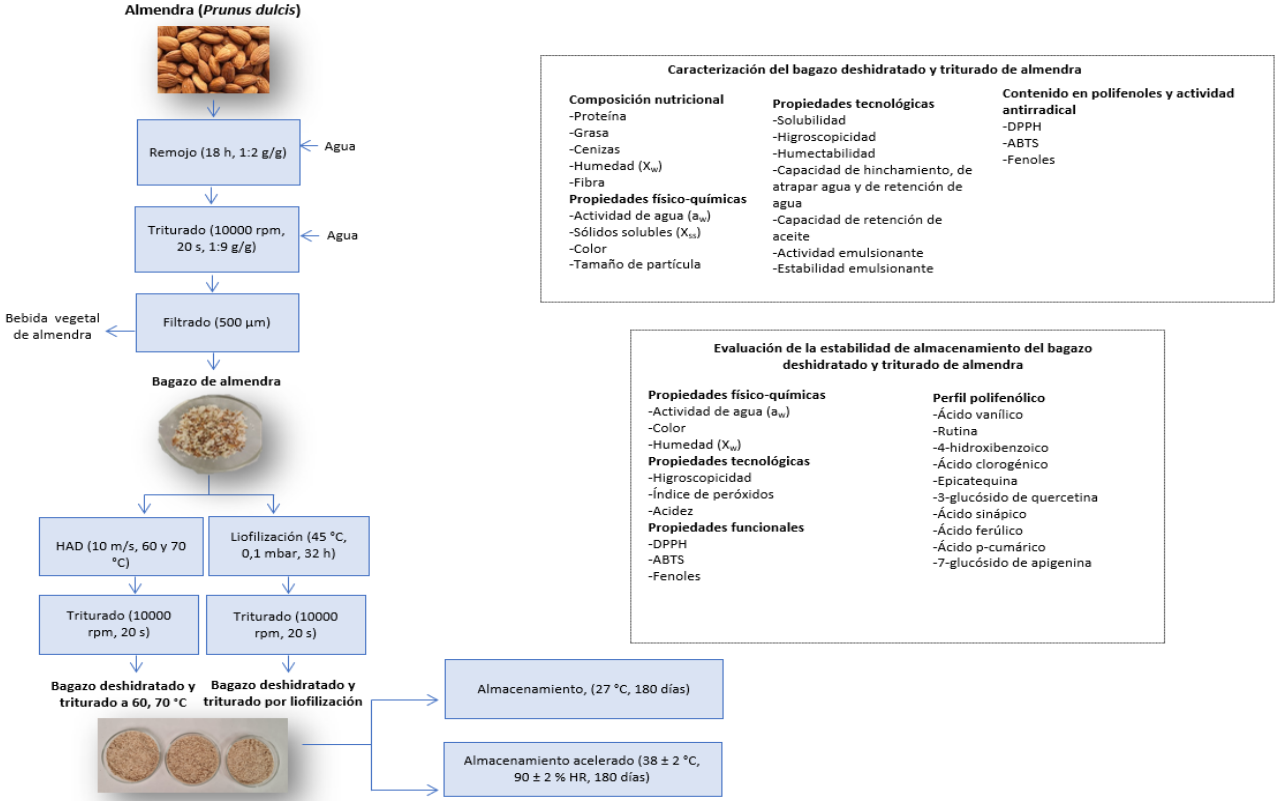

Figura 2.1. Diagrama del plan de trabajo para el procesado, deshidratación, almacenamiento y caracterización del bagazo de almendra fresco, y los bagazos deshidratados y triturados por aire calienta a 60 °C, 70 °C y liofilizados.

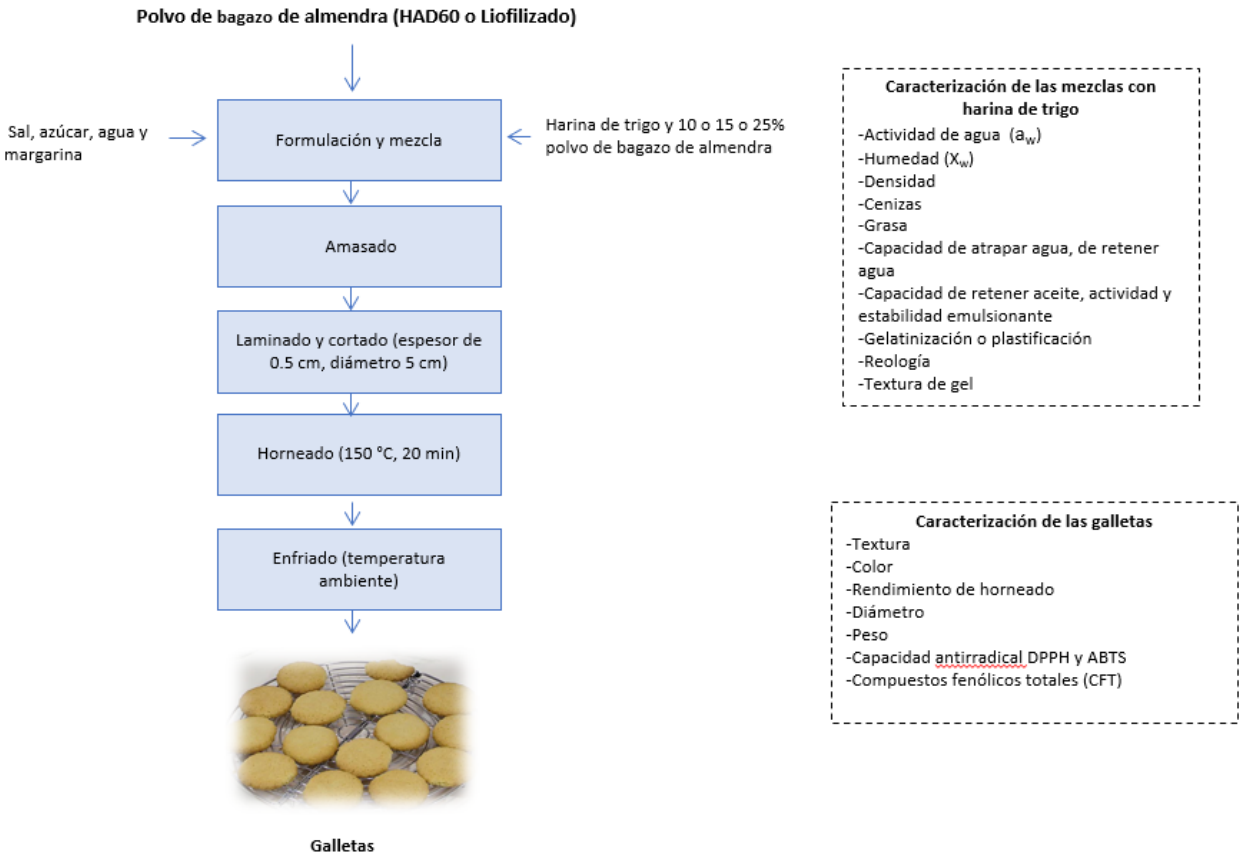

Figura 2.2. Diagrama del plan de trabajo para la formulación, preparación y caracterización de las galletas, que incluyen diferentes porcentajes (10%, 15% y 25%) de bagazo de almendra deshidratado a 60 °C y liofilizado.

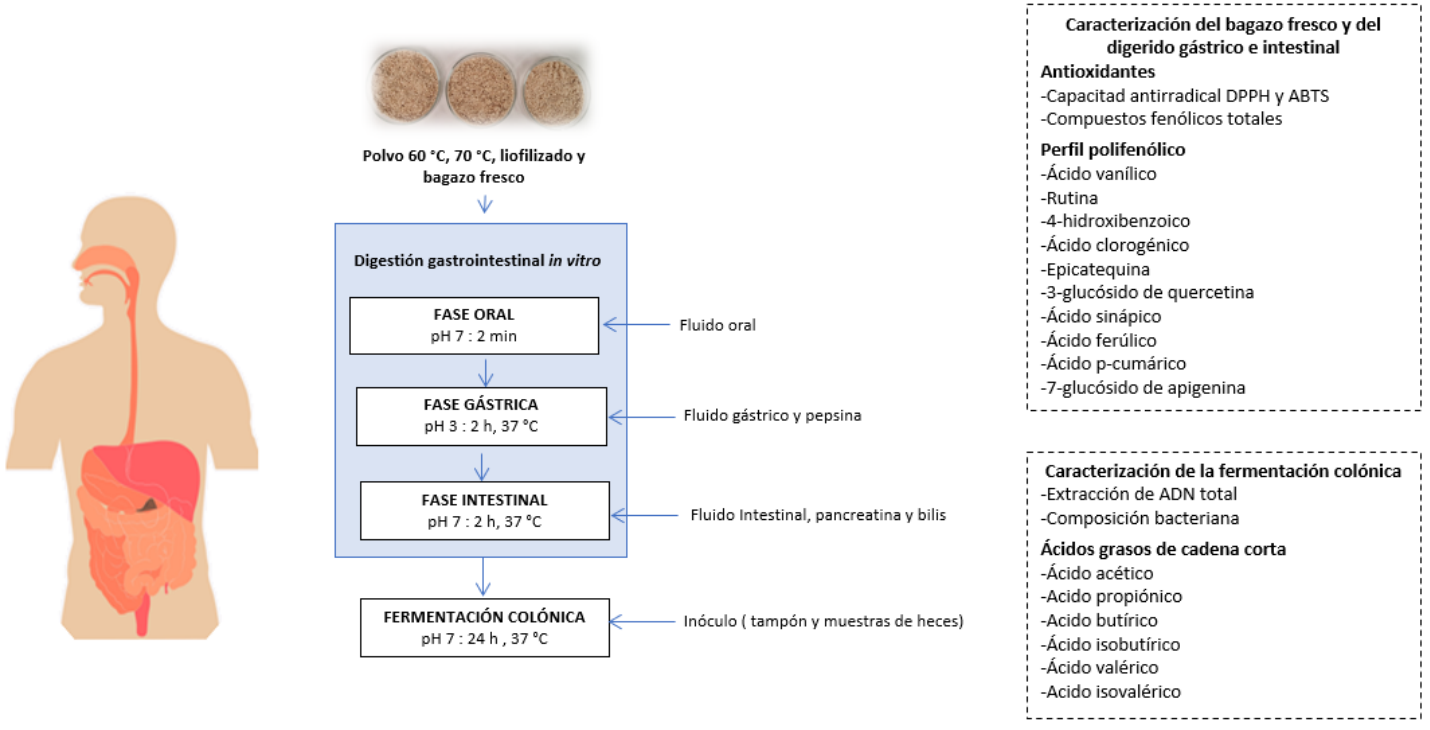

Figura 2.3. Diagrama del plan de trabajo para el proceso de digestión gastrointestinal *in vitro* y fermentación colónica de los bagazos de almendra, secados por aire caliente a 60 °C y 70° C, y liofilizados. Caracterización del digerido gástrico e intestinal, y de la fermentación colónica para la cuantificación de los ácidos grasos de cadena corta resultantes.

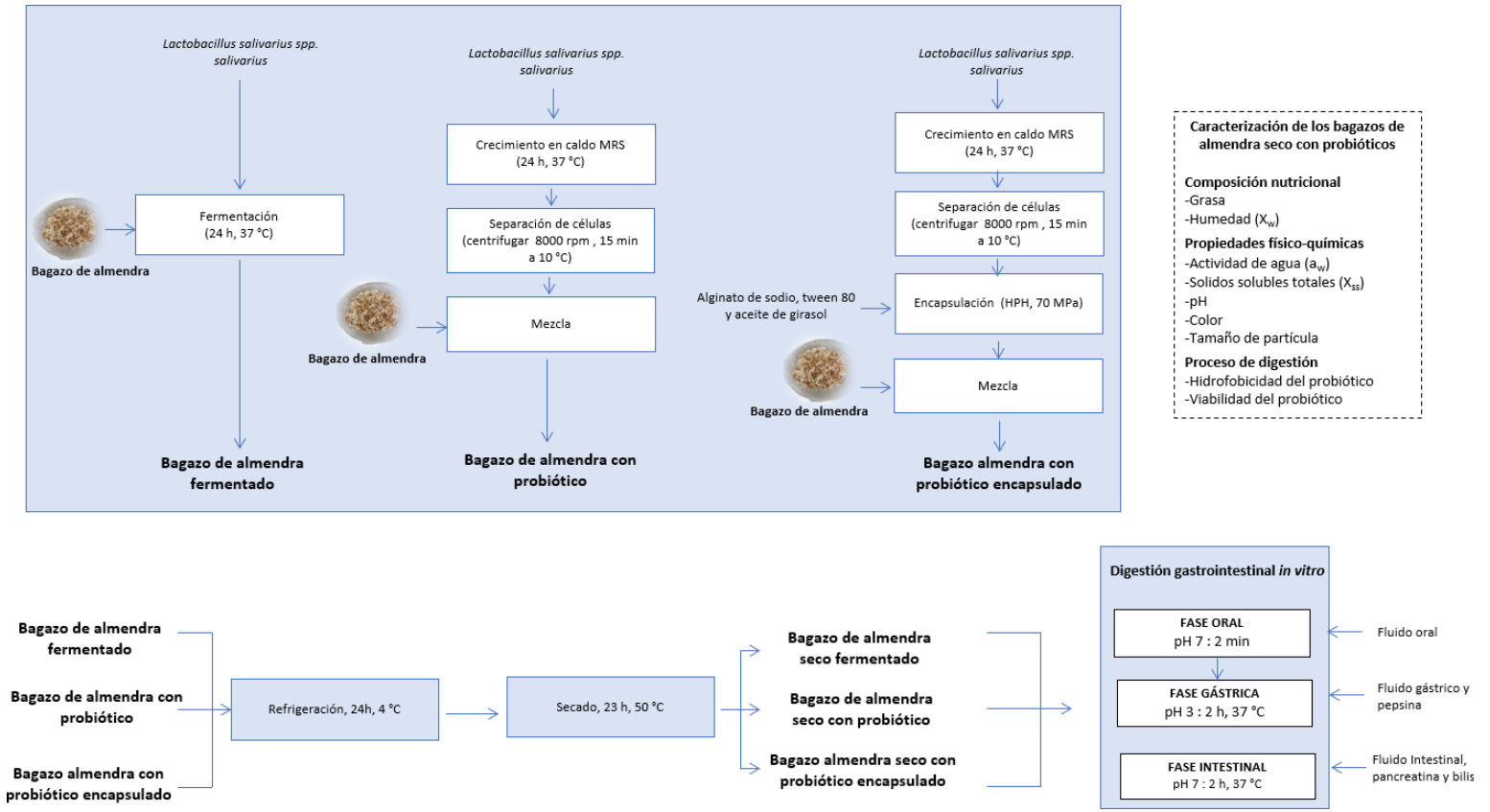

Figura 2.4. Diagrama del plan de trabajo para la incorporación del probiótico *Lactobacillus salivarius* spp. al bagazo de almendra fresco, y valoración de su resistencia al proceso de deshidratación y la digestión gastrointestinal *in vitro*.

3. RESULTADOS Y DISCUSIÓN

CAPÍTULO I

CAPÍTULO I: EL BAGAZO DE ALMENDRA COMO INGREDIENTE DE INTERÉS PARA LA INDUSTRIA ALIMENTARIA: ESTABILIZACIÓN, ALMACENAMIENTO Y APLICACIONES.

ARTÍCULO 1

Duarte, S., Betoret, E., Barrera, C., Seguí, L., & Betoret, N. (2023). Integral Recovery of Almond Bagasse through Dehydration: Physico-Chemical and Technological Properties and Hot Air-Drying Modelling. *Sustainability*, *15*(13), 10704. **DOI:** https://doi.org/10.3390/su151310704

ARTÍCULO 2

Duarte, S., Betoret, E., & Betoret, N. (2024). Shelf life of almond bagasse powders as influenced by dehydration method and storing conditions. **En revisión**, revista *Foods*.

ARTÍCULO 3

Duarte, S., Harasym, J., Sánchez-García, J., Betoret, E., & Betoret, N. (2024). Suitability of almond bagasse powder as a wheat flour substitute in biscuit formulation. **Enviado**, revista *Plant Food for Human Nutrition*.

RESUMEN CAPÍTULO I

La industria alimentaria enfrenta un gran desafío debido a la generación masiva de desperdicios, y al mismo tiempo, a la demanda de alimentos más saludables, ricos en proteínas, fibras y de origen vegetal. El bagazo de almendra, subproducto de la elaboración de la bebida vegetal de almendra, contiene antioxidantes, fibra, proteínas y un considerable contenido en grasa, lo que lo convierte en un prometedor ingrediente funcional para la industria alimentaria. Sin embargo, su estabilización y conservación con una calidad aceptable puede verse comprometida debido al alto contenido en grasa. Diferentes factores intrínsecos y extrínsecos favorecen reacciones de oxidación lipídica que pueden generar olores y sabores no deseados.

En este capítulo se estudia el impacto del secado por aire caliente a 60 °C y 70 °C y de la liofilización sobre las propiedades tecno-funcionales del bagazo de almendra. Se modelizaron las curvas de secado y se obtuvieron las isotermas de sorción. A continuación, se realizó un estudio de almacenamiento a temperatura ambiente y en condiciones de almacenamiento acelerado de los polvos obtenidos mediante secado por aire caliente y liofilización. Durante un periodo de 6 meses se realizó un seguimiento del crecimiento microbiológico, la acidez, el índice de peróxidos, la capacidad antirradical y el contenido en algunos polifenoles específicos. Por último, se evaluó la idoneidad del polvo como ingrediente sustitutivo en la elaboración de productos de panadería. Se caracterizaron, en términos de propiedades tecnológicas y reológicas, diferentes mezclas de harina de trigo conteniendo el 10%, 15% y 25% de polvo de bagazo de almendra obtenido por secado con aire a 60 °C aire o liofilización. También, se determinaron las propiedades físicas y antioxidantes de galletas con elevado valor nutricional y funcional elaboradas con las mezclas.

Los resultados se presentan en forma de tres artículos de investigación. En el primero de ellos se concluyó que tanto el secado por aire caliente como la liofilización resultaron ser operaciones adecuadas para estabilizar el bagazo de almendra. Las dos operaciones, combinadas con un triturado adecuado, proporcionaron polvos con propiedades favorables para su uso por la industria alimentaria. No obstante, el método de deshidratación aplicado afectó de manera significativa las propiedades del polvo final condicionando las aplicaciones más recomendables. En relación con los resultados más destacables, todos los polvos presentaron diferencias de color perceptibles para el ojo humano con respecto al bagazo fresco. En cuanto a las propiedades de interacción con el agua, se observó que los valores de solubilidad en agua variaron entre el 26,2% y el 30,1%, siendo más altos en las muestras liofilizadas, presumiblemente debido al daño estructural severo causado por la congelación y sublimación del agua durante este proceso. La higroscopicidad de los polvos se mantuvo por debajo del 17%, indicando una baja capacidad de absorción de agua. Por otro lado, en cuanto a la humectabilidad, la capacidad de hinchamiento, y la capacidad de absorber y retener agua, se observó que los polvos liofilizados presentaron valores más elevados en comparación con los polvos secados por aire caliente, debido sobre todo a la distribución del tamaño de partícula. En cuanto a las propiedades de interacción con el aceite, hubo diferencias notables entre los polvos liofilizados y los secados al aire, siendo los valores más altos para los polvos liofilizados. En este caso el daño estructural sufrido durante la operación puso a disposición más grupos hidrófilos e hidrófobos procedentes de moléculas complejas, mejorando las propiedades de interacción con el aceite. En relación con la actividad antirradical no se presentaron diferencias significativas entre las muestras deshidratadas, si bien el contenido en fenoles totales fue mayor en las muestras liofilizadas.

En el segundo estudio, los polvos deshidratados en las diferentes condiciones se colocaron en bolsas herméticas y se almacenaron a temperatura ambiente (27 ± 2 °C, 50-75% de humedad relativa) y en condiciones aceleradas (38 ± 2 °C, 90 ± 2% de humedad relativa) durante 6 meses. Se realizó un seguimiento del contenido en agua, la actividad de agua, la higroscopicidad, el crecimiento microbiológico, la acidez, el índice de peróxidos, la capacidad antirradical y el contenido en algunos polifenoles específicos mediante análisis cada 30 días para las muestras almacenadas a temperatura ambiente y cada 15 días para las muestras en almacenamiento acelerado. Los resultados mostraron un aumento significativo en la actividad de agua y la higroscopicidad si bien los resultados finales no resultaron críticos en ninguno de los casos. La higroscopicidad se mantuvo por debajo del 23% para todas las muestras, lo que se considera un nivel medio-bajo. La acidez también registró un aumento significativo desde el día 0 hasta el día 180, siendo más marcado en las muestras sometidas a almacenamiento acelerado, con incrementos del 73%, 61% y 25% para las muestras HAD60 AC, HAD70 AC y LYO AC, respectivamente. Los valores del índice de peróxidos aumentaron considerablemente durante el almacenamiento acelerado de 180 días, oscilando entre 2,4 y 24,6 O_2/kg. Este aumento se debe al incremento de temperatura (38 ± 2 °C), lo que conduce a la descomposición de los ácidos grasos y a la formación de hidroperóxidos. Al finalizar el periodo de almacenamiento, se observó un aumento en la capacidad antirradical por DPPH y ABTS, así como en el contenido de fenoles totales, especialmente notable en las muestras secadas por aire caliente y sometidas a almacenamiento acelerado. Este fenómeno podría atribuirse a los productos formados por reacciones de Maillard, los cuales presentan excelentes propiedades antioxidantes. Esta misma tendencia se reflejó en el contenido de polifenoles específicos, los cuales aumentaron significativamente al término del

periodo de almacenamiento tanto a temperatura ambiente como en condiciones de almacenamiento acelerado.

Finalmente, en el tercer estudio se evaluó la idoneidad del polvo como ingrediente sustitutivo en la elaboración de productos de panadería. Se caracterizaron, en términos de propiedades tecnológicas y reológicas, diferentes mezclas de harina de trigo conteniendo el 10%, 15% y el 25% de polvo de bagazo de almendra obtenido por secado con aire a 60 °C o por liofilización. También, se determinaron las propiedades físicas y antioxidantes de galletas elaboradas con las mezclas. Los resultados más relevantes mostraron cambios significativos en la capacidad de retención de aceite y en la estabilidad y actividad emulsionante. Estos cambios estuvieron vinculados tanto al porcentaje de sustitución de harina de trigo como al método de secado empleado para obtener el polvo de almendra. Por otro lado, se observaron diferencias significativas en la capacidad de retención y absorción de agua cuando se utilizó polvo de almendra obtenido por métodos de deshidratación diferentes, registrándose valores más altos en las mezclas que contenían polvo liofilizado. Esto se atribuyó a que estas propiedades están influenciadas por el tamaño de partícula, resultando mejores características de interacción con el agua en las mezclas con polvo liofilizado. La ausencia de almidón y el elevado contenido en grasa y fibra insoluble del polvo de bagazo de almendra determinó el comportamiento viscoelástico de las mezclas hidratadas; a medida que aumentó el porcentaje de sustitución con polvo de bagazo de almendra, la viscosidad final disminuyó. También lo hicieron los módulos viscoso y elástico obtenidos en un ensayo dinámico oscilatorio. Sin embargo, los resultados obtenidos sugirieron que no hubo cambios significativos en las características estructurales del gel formado al mezclar la harina de trigo con el polvo de bagazo de almendra. Es decir, la estructura procedente de las moléculas de almidón y las proteínas de la harina de trigo siguió

determinando el comportamiento durante el amasado de la mezcla hidratada, aunque esto se tradujo en una masa más blanda conforme aumentó el porcentaje de sustitución. Finalmente, cabe remarcar que el uso de las mezclas para la elaboración de galletas proporcionó un producto final con mayor contenido en componentes antioxidantes.

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Integral Recovery of Almond Bagasse through Dehydration: Physico-Chemical and Technological Properties and Hot Air-Drying Modelling

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ARTÍCULO 1: Integral recovery of almond bagasse through dehydration: Physico-chemical and technological properties and hot air-drying modelling.

ABSTRACT

Recovering waste from industrial food processes and developing new healthy foods as plant protein sources has been a major focus of scientific research and industrial innovation in food. Thus, the consumption of plant-based beverages from soy, oat, or almond has been promoted. In the case of almonds, the resulting solid bagasse has an interesting nutritional profile and its transformation into a powdered product could be a valuable option for the food industry. The main objective of this work was to determine the effect of hot air drying at 60 and 70 °C and freeze-drying on the physicochemical, water interaction, emulsifying and antioxidant properties of powdered almond bagasse. Furthermore, hot air-drying curves have been modelled and isotherms at 20 °C have been performed. The proximate composition of the powder revealed a protein content of 15% and a fat content of 25%, which makes it a remarkably different powder from those obtained from other vegetable residues such as fruits and vegetables. This composition was decisive in the effect of the drying method and drying temperature, and no significant differences were observed on the physico-chemical or antioxidant properties regardless of the drying method used. However, freeze-drying resulted in a powder with a more homogeneous particle size distribution and better oil-interaction properties, especially with higher emulsifying activity and stability.

Keywords: plant-based almond drink; almond; solid bagasse; air drying; freeze drying; sorption isotherms

1. Introduction

The food industry has become increasingly aware of the impact of food waste in economics and environment, and the need to reduce it. In fact, primarily motivated by the fulfilment of the Sustainable Development Goals (SDG), 71% of Spanish companies have a defined internal strategy to fight against food waste [1]. The production of new functional ingredients, biofuel production, or bioactive compounds extraction are some of the most considered strategies for food processing residues valorization [2–4]. The composition profile of the residues and their physico-chemical properties must be known in order to identify the opportunity for revalorization and to determine the possible uses [5].

The new trends in food development have been defined in the last years by the increased consumer awareness for health and sustainability and the growing incidence in allergies or food intolerances. Thus, the consumption of plant-based food has been promoted. Plant-based beverages or vegetable drinks are a clear example of this new orientation; more weight is being put behind them as an alternative to the consumption of dairy drinks. Among these, vegetable drinks such as soy, oats, rice, almond, and coconut stand out. In the manufacturing process, the raw material is soaked in water, milled, and filtered, resulting in a liquid phase that will constitute the vegetable beverage. The remaining solid material is usually referred to as press cake or bagasse, and it is usually discarded or used for animal feed or as fertilizer [6].

Regarding the almond, a relevant area is dedicated to its cultivation in Spain, only behind the olive and the grape [7]. Its consumption as a nut is growing due to the healthy properties associated with its unsaturated fatty acids (56%), proteins (23%), fiber (11%) and other carbohydrates (7%), minerals, and vitamins content [8].

Additionally, and motivated by new consumer trends, it is being increasingly used as the raw material for obtaining vegetable almond drink. The resulting solid bagasse has an interesting nutritional profile, which makes it very attractive for valorization. Its transformation into a powdered product with good nutritional properties for use as a functional food ingredient could be an option [9,10]. Determining its physico-chemical, technological, and functional properties is essential in determining its best use.

The main objective of this study was to determine the effect of hot air-drying at 60 and 70 °C and freeze-drying on the physico-chemical, water interaction, emulsifying, and antioxidant properties of powdered almond bagasse. Furthermore, hot air-drying curves have been modelled and isotherms at 20 °C have been performed.

2. Materials and Methods

2.1. Process for Obtaining Almond Bagasse and Almond Bagasse Powder

Natural peeled almonds were purchased from a local supermarket and ground with tap water in a ratio of 1/9 (w/w). A domestic food processor (Thermomix®, Vorwerk, Spain) at 10,000 rpm for 20 s was used. The grind was then filtered with a stainless steel 500 µm sieve and the almond bagasse was recovered for further characterization and processing. The recovered bagasse mass was about 82% of the rehydrated kernel mass.

For obtaining the dried almond bagasse, the moist almond bagasse was distributed homogeneously in plastic grids with a nominal opening of 2 mm and then introduced into the dryer until a water activity (aw) below 0.3 was reached. A convective dryer (Pol-eko Aparatura, Katowice, Poland) with cross-flow air at a

velocity of 10 m/s at 60 or 70 °C for 10 h and 7 h, respectively, was used to obtain air dried (HAD) bagasse, and a freeze-dryer (Telstar, Lioalta-g) was used to obtain the freeze-dried (LYO) one from almond bagasse previously frozen at −40 °C for 24 h. The inlet air to the convective dryer was ambient air at 25 °C and 25% of relative humidity. After that, the dried almond bagasse was ground using a food processor (Thermomix®, Vorwerk, Spain) at 4000 rpm for 20 s in intervals of 5 s and then at 10,000 rpm for 20 s in intervals of 5 s, thus obtaining almond bagasse powders with coarse granulometry. Finally, the powders were stored at 20 °C in light-opaque glass jars to prevent deterioration and oxidation reactions.

During the hot air-drying experiments, the samples weight change was registered. The evolution of the moisture content was determined from the initial moisture content and the mass of the samples at each time. Plotting the moisture on dry basis versus time made it possible to graph the drying curves and, from these, the drying rate curves. Data were modeled according to a lineal empirical and diffusional models. The goodness of fit was assessed by the coefficient of determination (R^2) (Equation 3.1), the root mean square error (RMSE) (Equation 3.2), and the mean relative error (MRE) (Equation 3.3). For the best fit, the R^2 should be high and RMSE and MRE should be low.

$$\mathrm{R}^2 = 1 - \frac{\sum_{\mathrm{i}=1}^{\mathrm{n}} \left(x_{exp,i} - x_{pred,i}\right)^2}{\sum_{\mathrm{i}=1}^{\mathrm{n}} \left(x_{exp,i} - \overline{x}\right)^2} \qquad (3.1)$$

$$\mathrm{RMSE} = \frac{\sqrt{\sum_{\mathrm{i}=1}^{\mathrm{N}} (x_{exp,i} - x_{pred,i})^2}}{\mathrm{N}} \qquad (3.2)$$

$$\mathrm{MRE} = \frac{1}{\mathrm{N}} \sum_{\mathrm{i}=1}^{\mathrm{N}} \frac{\left|x_{exp,i} - x_{pred,i}\right|}{x_{exp,i}} \qquad (3.3)$$

Where x represents the variable under consideration, i.e., the velocity in the linear model and the reduced driving force in the diffusional model; $\overline{x}$; represents the mean value; N is the number of determinations; Exp.; experimental. Pred: predicted by the model.

2.2. Analytical Determinations

The water activity (a_w) of almond bagasse and almond bagasse powders (air dried at 60 °C and 70 °C and freeze-dried) was determined with a dew point hygrometer (DECAGÓN Aqualab 4TE) at 20 °C. **The moisture content** was determined following the official method in dried fruits established by the AOAC [11]. **The total soluble solids** (TSS) were determined by refractometry. For this, a dilution of the sample in distilled water was carried out in a ratio of 1:10 (m/v) and the Brix degrees were measured by means of a refractometer (ABBE ATAGO 3-T) thermostated at 20 °C. The **fat** content of almond bagasse was determined by Soxhlet extraction with petroleum ether according to the method established by the AOAC [12]. A relation of 5 g sample/90 mL solvent at 290 °C was used. The **protein** content was determined by the Kjeldahl method, considering 5.18 as the conversion factor from N to protein [13]. Different Van Soest **fibre** fractions, including neutral detergent fibre, which corresponds to the lignin, cellulose, and hemicellulose contents (NDF), acid detergent fibre, which corresponds to the lignin and cellulose contents (ADF), and lignin with acid detergent, which corresponds to the pure lignin content (LDF), were determined [14]. The values were used to estimate hemicellulose, cellulose, and lignin content. The **ash** determination was carried out by incineration of the material in a muffle at 550 °C [15].

2.3. Water Interaction and Emulsifying Properties

The **solubility** (SD) was determined following the procedure described by Mimouni et al. [16], in which the mass fraction of a dissolved solid (SS) in a rehydrated sample (TS) is determined. The **hygroscopicity** was evaluated according to the method described by Cai and Corke. [17]; 0.5 g of each sample was weighed in glass crucibles and taken to an airtight chamber next to a saturated solution of sodium sulphate (Na_2SO_4) for 7 days at 25 °C. **Wettability**, defined by the time it takes for a sample to become wet in its entirety, was determined by weighing 2 g of each powder sample slowly poured into a beaker with 20 mL of distilled water, and measuring the time it took to become fully wet [18]. The **swelling capacity** (CS) was obtained from the ratio between volume occupied by 1 g of sample and that after hydration for 18 h at 25 °C [19,20]. **Water holding capacity** (WHC) is defined as the amount of water retained by the sample without applying any external force. It was determined by measuring the water content of the precipitate after mixing 0.2 g of sample and 10 mL of distilled water and left to stand for 18 h at 25 °C [19]. **Water retention capacity** (WRC) is defined as the ability of a sample to retain water after being subjected to an external force such as the centrifuge [19]. For its determination, 1 g of sample was weighed in a graduated conical tube and 10 mL of distilled water was added and left to stand for 18 h at 25 °C. After this time, it was centrifuged for 30 min at 2000 rpm, the supernatant was removed, and the sedimented residue was weighed. The **oil retention capacity** was evaluated following the methodology proposed by Garau et al. [21]. First, 0.2 g of sample and 1.5 g of sunflower oil were mixed and left to stand overnight at 20 °C. After that, the mix was centrifuged at 3416 rpm for 5 min, and with a Pasteur pipette the supernatant was removed and the weight of the residue was obtained. The oil retention capacity was evaluated based on the increase in the weight of the sample, and the results were

expressed in g of oil absorbed by g of the initial sample. The **emulsifying activity** was determined following the methodology proposed by Yasumatsu et al. [22]. To carry out the procedure, a 2% (*w/v*) sample-water solution was prepared. Next, 7 mL of this solution was mixed with 7 mL of sunflower oil and homogenized for 5 min in a vortex at 2400 rpm. Finally, it was centrifuged at 10,000 rpm for 5 min and the volume of the emulsion formed was calculated by the ratio between the emulsion volume and the total fluid volume**. Emulsifying stability** was determined following the methodology proposed by Yasumatsu et al. [22]. For this, a 2% (*w/v*) sample-water solution was prepared. Then, 7 mL of this solution was mixed with 7 mL of sunflower oil and homogenized for 5 min in a vortex at 2400 rpm. Finally, it was heated to 80 °C for 30 min, allowed to cool, and centrifuged at 2000 rpm for 5 min. Emulsifying stability was calculated as the ratio between the emulsion volume and the total fluid volume.

2.4. Particle Size

The particle size of almond bagasse powders was determined by the wet method. Laser diffraction equipment (Masterizer, Malvern Instruments Limited, Worcester, UK) with a measurement range between 0.02 and 200 microns equipped with a blue light of 470 nm wavelength was used. A small amount of sample was diluted in deionized water until reaching an obscuration of 8–9%. Finally, the particle size distribution was obtained and was characterized by the mean diameter of equivalent volume (D [4,3]), equivalent diameter calculated from the area of the particles (D [3,2]), and, finally, d90, d50 and d10, representing the percentiles of the distribution, i.e., the volume of particles below 90%, 50%, and 10% of the particles analysed, respectively.

2.5. Optical Properties

The CIE*L*a*b* coordinates were measured with a spectrocolourimeter (MINOLTA, CM-3600D, Japan), considering the standard light source D65, the 10° standard observer, and the surface reflectance spectra between 400 and 700 nm. The chroma (Cab) and the colour differences (ΔE) of the powders compared to almond bagasse were calculated using Equations 3.4 and 3.5, respectively.

$$C_{ab} = \sqrt{a^2 + b^2} \quad (3.4)$$

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (3.5)$$

2.6. Antiradical Capacity and Total Phenols Content

For the extraction of phenols and other components with antiradical capacity, a methanol-water mixture 80:20 (*v*/*v*) was prepared and used as a solvent in the relation 1 g sample/100 mL solvent. After 1 h of magnetic stirring, the mix was centrifuged (Selecta, "Medrifriger BL-S") at 10,000 rpm for 5 min at 20 °C. Determinations were made on the supernatant, hereinafter referred to as extract.

2.6.1. Total Phenol Content

The determination of total phenols was performed following the colorimetric method of Folin–Ciocalteu [23]. In a spectrophotometry bucket, 0.125 mL of extract, 0.125 mL of the Folin–Ciocalteu reagent (Sigma-Aldrich) and 0.5 mL of bidistilled water were added in that order and allowed to react for 6 min. After this time, 1.25 mL of 7% (*m*/*v*) sodium carbonate solution and 1 mL of distilled water were added. As a reference, a target was used where the sample was replaced by bidistilled water and allowed to react for 90 min. Finally, the absorbance was measured at 765 nm in

a spectrophotometer (Thermo Scientific, Helios Zeta U/Vis). The results obtained were compared to a standard curve of gallic acid (purity ≥ 98%, Sigma-Aldrich) and expressed as mg of gallic acid equivalents/g of dry matter (mg GAE/g dm).

2.6.2. Antiradical Capacity by DPPH and ABTS Methods

The antioxidant capacity was determined following the DPPH method described by Stratil et al. [24] with some modifications. First, 0.1 mL of the extract and 2.9 mL of the methanol-DPPH solution (0.394 of DPPH reagent/mL methanol) were mixed and the absorbance was measured at 517 nm in a spectrophotometer (Thermo Scientific, Helios Zeta U/Vis). The results were expressed as mg of trolox equivalent/g of dry matter (mg TE/g dm) using the Trolox calibration line ($C_{14}H_{18}O_4$, purity ≥ 7%, Sigma-Aldrich) as the reference standard antioxidant, for the range of concentrations between 0 and 500 mg/L.

The antioxidant activity was also evaluated by the ABTS radical method (2,20-azobis-3-ethyl benzothiazolin-6-sulfonic acid) [25]. A solution including the radical ABTS 7 mM and potassium persulfate 2.45 mM in distilled water was prepared and incubated in darkness at room temperature for 16 h. Once this time had elapsed, a dilution with phosphate buffer was carried out to reach an absorbance of 0.7 ± 0.02 at 734 nm. Then, in a spectrophotometry bucket, 0.1 mL of extract with 2.9 mL of ABTS solution was reacted. As a reference, a white where the sample was replaced by bidistilled water was prepared. The absorbance was measured after 0, 3 and 7 min of reaction at a wavelength of 734 nm in a spectrophotometer (Thermo Scientific, Helios Zeta UV/Vis). The results were expressed as mg of trolox equivalent/g of dry matter (mg TE/g dm), using the Trolox calibration line ($C_{14}H_{18}O_4$, purity ≥ 7%, Sigma-Aldrich) as the reference standard antioxidant for the range of concentrations between 0 and 500 mg/L.

2.7. Sorption Isotherms

Sorption isotherms were determined following the gravimetric method described by Wolf et al. [26]. This method uses saturated salt solutions to keep a known and controlled humidity environment within a closed vessel at specific temperature conditions. First, 1 g of sample was placed in a closed jar at 20 °C together with one of the next saturated salt solutions: LiCl (a_w = 0.1), CH_3COOK (a_w = 0.23), $MgCl_2$ (a_w = 0.32), K_2CO_3 (a_w = 0.43), Mg $(NO_3)_2$ (a_w = 0.52), NaCl (a_w = 0.75), KCl (a_w = 0.85), and $BaCl_2$ (a_w = 0.90). The samples were weighed every eight days until a constant weight was reached.

2.8. Statistical Analysis

The results were statistically analysed with Statgraphics software (Centurion XVI.I, Statpoint Technologies, Inc., Warrenton, VA, USA) at a 95 % confidence level (p-value ≤ 0.05). The normality of the data was tested with the Shapiro–Wilk test (p > 0.05). The data were processed by simple ANOVA after checking the normality of the data. For each processing treatment, three different experiments with three replicates each were carried out. Significant differences (p-value < 0.05) among groups were determined by Fisher's LSD test.

3. Results and Discussion

3.1. Properties of Almond Bagasse Powders

Table 3.1 shows the composition, physico-chemical, water interaction, emulsifying properties, and colour of fresh, air dried, and freeze-dried almond bagasse powders. Dehydration, in all cases, reached a water activity lower than 0.3, which is the recommended limit to ensure the stability of powdered products

[27,28]. Although the water activity limit for microbial growth is 0.90 for most bacteria and 0.87 and 0.75 for most yeasts and fungi, a water activity limit lower than 0.3 assures kinetic stability in powdered products since it is guaranteed that there is no free water that can participate in chemical and enzymatic reactions. The moisture content in the final samples was low, as isotherms showed a very low water binding capacity (see isotherms section). Thus, more than 98% of the water is easily removed during drying.

Considering the fat and protein content, the almond bagasse retained a high percentage of fat and protein from fresh almond and there were no significant differences between fresh and dehydrated samples. Fat content remained around 25% (0.25 g/g_{dm}) in the bagasse and protein reached 16–17% (0.16–0.17 g/g_{dm}); the initial values in fresh almond were around 54% and 25%, respectively [29]. In a study carried out with fresh baru almond, fat content around 39–43% was reported, and the protein content was around 23–28%, slightly higher than those obtained for almond bagasse [30]. Compared with other cereal by-products of interest to the food industry, protein content was similar to those obtained in rice bran (0.14 g protein/g) [31], oat bran (0.17 g protein/g) [32], by-product from tofu (0.15 g protein/g) [33], and soybean residue (0.15 g protein/g) [33], but lower than that for fresh okara (0.39 g protein/g) [34] and rice bran (0.22 g protein/g) [35]. Particular attention should be paid to the fat content. Stability in low-moisture, fat-containing foods is highly dependent on the characteristics of the matrix, its microstructure, and the presence of other macronutrients such as protein. Oxidation mechanisms are complex and need to be studied on a case-by-case basis to ensure proper packaging and storage [36].

According to the fibre content, no significant differences among treatments or fresh almond bagasse were detected. Van Soest fibre includes cellulose and lignin as insoluble fraction and the hemicellulose as a more soluble one. Values of total fibre were above those reported for okara fresh matter (13.84 g/100 g) [37], rice bran (28.6 g/100 g) [35], and oat bran (15.55 g/100 g of dry matter) [32], but they were below those of other by-products, such as by-product from tofu (58.6 g/100 g of dry matter) [33], bran fibre rice (53.25 g/100 g of dry matter) [35], or solid by-product tiger nut (59.71 g/100 g) [38], and similar to carrot skin (45.45–49.23 g/100 g of dry weight) [39]. Soluble dietary fibres such as pectin cannot be quantified by the fibre determination method used. However, this fraction could be estimated by the difference between the total mass and the total of the macronutrients considered. As the sum of fat, protein, fibre and water is 100%, the more soluble fibre including pectin can be considered negligible. Almond bagasse powders could be used as ingredients promoting intestinal transit more than an ingredient conferring viscosity since high content in soluble than insoluble was fibre observed in all cases. Soluble fibre is the one that confers viscosity properties, ability to form gels, and emulsifying capacity, while insoluble fibre with a greater porosity and lower density promotes intestinal transit [40].

Table 3.1. Composition, physico-chemical, water interaction, emulsifying properties and colour of fresh almond bagasse and, air dried (HAD60: hot air dried at 60 °C; HAD70: hot air dried at 70 °C) and freeze-dried (LYO) almond bagasse powders. The values in brackets for fresh bagasse refer to the composition expressed in g/g of raw material. Mean ± standard deviation of three repetitions. Different superscripts letters in the same line indicate statistically significant differences with a confidence level of 95%. $_{dm}$, dry matter; $_{w}$, water; X_w, water content; X_{ss}, soluble solids content; WHC, water holding capacity; WRC, water retention capacity.

	FRESH	HAD60	HAD70	LYO	p-value
Physico-chemical properties					
a_w	0.99 ± 0.08a	0.23 ± 0.04^{b}	0.20 ± 0.06^{bc}	0.13 ± 0.02^{c}	0.0000
Fat (g/g_{dm})	0.25 ± 0.002^{a} (0.11)	0.252 ± 0.002^{a}	0.253 ± 0.004^{a}	0.250 ± 0.006^{a}	0.7106
Protein (g/g_{dm})	0.15 ± 0.03^{a} (0.07)	0.16 ± 0.04^{b}	0.16 ± 0.03^{b}	0.165 ± 0.008^{b}	0.0030
X_w (g/g_{dm})	1.262 ± 0.011^{b} (0.558)	0.014 ± 0.002^{a}	0.015 ± 0.012^{a}	0.02 ± 0.08^{a}	0.0000
Ashes (g/g_{dm})	0.031 ± 0.011^{a} (0.014)	0.031 ± 0.007^{a}	0.03 ± 0.06^{a}	0.030 ± 0.012^{a}	0.0000
Fiber Van Soest (g/g_{dm})	0.47 ± 0.02^{a} (0.21)	0.45 ± 0.02^{a}	0.50 ± 0.03^{a}	0.50 ± 0.03^{a}	0.6605
Cellulose and lignine (g/g_{dm})	0.17 ± 0.02^{a} (0.08)	0.20 ± 0.05^{ab}	0.20 ± 0.15^{ab}	0.21 ± 0.02^{b}	0.0005
Hemicellulose (g/g_{dm})	0.23 ± 0.04^{a} (0.10)	0.260 ± 0.014^{a}	0.290 ± 0.012^{a}	0.295 ± 0.002^{a}	0.0008
X_{ss} (g_{ss}/g_{dm})	0.013 ± 0.003^{a} (0.006)	0.013 ± 0.004^{a}	0.013 ± 0.004^{a}	0.014 ± 0.004^{a}	0.6810
Water interaction properties					
Solubility (%)	-	29 ± 1^{b}	26.2 ± 2.2^{a}	30.1 ± 1.1^{c}	0.0000
Hygroscopicity (g_w/g)	-	0.17 ± 0.06^{a}	0.17 ± 0.17^{a}	0.17 ± 0.03^{a}	0.9763
Wettability (s)	-	8.3 ± 1.1^{a}	8.9 ± 0.6^{a}	8.3 ± 1.1^{a}	0.7458
Swelling capacity (mL_w/g)	-	4.51 ± 0.08^{a}	4.51 ± 0.08^{a}	4.51 ± 0.08^{a}	1.0000
WHC (g_w/g_{dm})	-	2.9 ± 0.5^{a}	2.6 ± 0.2^{a}	8.4 ± 1.8^{b}	0.0009
WRC (g_w/g_{dm})	-	4.5 ± 0.2^{a}	4.6 ± 0.2^{a}	5.91 ± 0.08^{b}	0.0000

Oil interaction properties					
Oil retention ability (g_o/g_s)	-	2.3 ± 0.5[a]	2.6 ± 0.2[a]	4.2 ± 0.06[b]	0.0047
Emulsifying activity (%)	-	19 ± 2[a]	20 ± 2[a]	34 ± 2[b]	0.0002
Emulsifying stability (%)	-	20 ± 2[a]	24 ± 2[a]	59 ± 2[b]	0.0000
Colour					
L	73.68 ± 0.07[a]	62.358 ± 0.010[c]	58.236 ± 0.002[d]	66.561 ± 0.001[b]	0.0010
a*	4.88 ± 0.02[d]	4.999 ± 0.009[c]	6.487 ± 0.009[a]	6.039 ± 0.002[b]	0.0039
b*	11.62 ± 0.04[d]	14.279 ± 0.006[c]	16.143 ± 0.017[a]	15.026 ± 0.014[b]	0.0030
C	12.61 ± 0.05[d]	15.128 ± 0.08[c]	17.398 ± 0.014[a]	16.194 ± 0.012[b]	0.0204
ΔE	-	11.625 ± 0.010[b]	16.167 ± 0.003[a]	7.971 ± 0.06[c]	0.0001

Water solubility values ranged from 26.2% to 30.1%, without significant differences between hot air-dried and freeze-dried samples. The freeze-dried samples showed slightly high solubility levels, presumably caused by the more severe structural damage induced by the freezing and subsequent water sublimation during lyophilization. An increase in the air-drying temperature resulted in a decrease in solubility, probably due to physical changes affecting macromolecules during the drying process. These physical changes during hot air drying could promote the formation of a surface crust, which can hinder the interaction between molecules and water [41]. Solubility values were lower when compared to those from other fruit powders such as passion fruit (44.6% to 57.56%) [42] or pineapple juice powder (81.56%) [43]. Nevertheless, the results were closer to those obtained for oat bran (ranging from 11.70% to 26.32% depending on the drying process applied) [44]. Clearly, a higher percentage of macromolecules such as insoluble fibre and proteins in the composition of by-products such as bran or bagasse provides lower solubilities. Additionally, the presence of a high percentage of fat makes the interaction with water molecules even more difficult.

Hygroscopicity is the capacity of a material or powder to absorb moisture and come into equilibrium with the relative humidity of the environment. The low water content of food powders could contribute to their high hygroscopicity, which gives rise to sticky and caked powders with low porosity, therefore decreasing their ability to rehydrate and retain aromas [45]. Food powder is considered good if it has low hygroscopicity [45]. According to Callahan et al. [46], a material can be considered non-hygroscopic when an increase of less than 20% (w/w) in moisture content above 90% relative humidity is observed after one week. Almond bagasse powder gained 0.17 g of water/g (17%) when equilibrated at 97% relative humidity after one week

and was therefore non-hygroscopic. Non-significant differences were observed among the samples.

Wettability, swelling capacity, the water holding capacity (WHC), and the water retention capacity (WRC) are largely conditioned by the particle size and composition, mainly the fibre type and fat content. Wettability and the swelling capacity of almond bagasse powders were not significantly affected by the drying method or air temperature. However, WHC and WRC are significantly higher in the lyophilized powders. A different size distribution (Figure 3.1) with a single peak indicative of a larger volume of larger particles could be the explanation for these differences. According to Bai et al. [44], the larger the particle size, the higher the wettability since water molecules can permeate through the larger voids left between the particles. Regarding composition, soluble fibre has a high capacity to retain water and expand to form a viscous solution, while insoluble fibre can also absorb and retain water in its fibrous matrix but in a lower quantity; fat, on the other hand, hinders any interaction with water. Lecumberry et al. [47] reported results for WRC in apple and orange pectin of 16.51 ± 3.77 and 28.07 ± 5.37 g water/g dry matter, respectively), these results being higher than those obtained for almond bagasse, which is consistent with the higher content of insoluble fibre and the presence of fat in the almond bagasse. Nevertheless, similar results were obtained in lulo bagasse (8.2 ± 0.7), a material also with a high content of insoluble fibre [48]. Bai et al. [44] provided data on WHC in oat bran (5.95 to 6.48 g of water/ g of dry matter), which were similar to those obtained in freeze-dried almond bagasse and slightly lower in hot air-dried powders.

Regarding oil interaction properties, the results obtained for almond bagasse powders showed good emulsifying properties, such as emulsifying activity and

emulsifying stability. Significant differences were detected between freeze-dried and air-dried samples, regardless of air temperature. The values obtained for freeze-dried powders were higher (Table 3.1). The emulsifying capacity is associated with the presence of hydrophilic and hydrophobic groups. The high protein content present in the almond bagasse justifies its good oil-interaction properties. In freeze-dried samples, the increased structural damage caused by freezing and sublimation contributes to the breakdown of complex molecules, leaving more hydrophilic and hydrophobic groups available for interaction and consequently improving the oil-interaction properties [49]. Regarding oil retention ability, similar values were reported for commercial fibres from lemon, orange, peach, apple, and persimmon (2.5 to 2.9 g oil/ g sample) [50]. Similar emulsion stability to that of freeze-dried almond bagasse powder was obtained for peas (59.4% ± 1.0) and lentils (55.0% ± 2.5). The emulsifying activity of almond bagasse could be compared with the results obtained for peas (40.9% ± 0.7) and lentils (39.9% ± 1.0) [51].

Associated with browning and oxidation reactions, all samples experienced colour differences when compared to the fresh almond bagasse (Table 3.1). These changes gave the samples more yellowish-red tones, denoted by higher values of the a* and b* coordinates. The saturation (C) in all cases shows a low value, being lower in the fresh bagasse. According to Bodart et al. [52], colour differences are imperceptible to the human eye when they are $\Delta E < 1$. Small differences can be seen when $1 < \Delta E < 3$ and will be visibly evident when the value of $\Delta E > 3$. Since in all samples the values were higher than 3, the changes were clearly perceptible. However, the colour difference in the freeze-dried powder was smaller since freeze-drying occurs under vacuum and at low temperatures, minimizing oxidation processes.

Figure 3.1 shows the particle size distribution and characteristic parameters of hot air-dried and freeze-dried almond bagasse powders. Practically, a monomodal distribution for the freeze-dried powder and a bimodal distribution for air-dried powders were observed. In the freeze dried, the structural breakdown induced by/100 g water freezing and sublimation resulted in a more homogeneous particle size distribution and a slight shift in the maximum towards a smaller particle size. Probably, in hot air-dried samples, phase transitions in macromolecules such as carbohydrates and proteins, and their different mechanical resistance to crushing, resulted in a more heterogeneous distribution, and specifically a bimodal one. This distribution is quite common in carbohydrate and fibre-rich powders produced by hot-air drying, such as blueberry powder and the tangerine skin powder dried at 70 °C [53,54].

	D [4,3]	D [3,2]	d (0.1)	d (0.5)	d (0.9)
HAD60	112 ± 3^{a}	25.8 ± 0.3^{a}	12.3 ± 0.1^{a}	81 ± 1^{a}	264 ± 9^{a}
HAD70	120 ± 4^{ab}	26.4 ± 0.3^{a}	12.6 ± 0.1^{a}	81 ± 2^{a}	291 ± 12^{b}
LYO	125 ± 11^{b}	26.0 ± 0.8^{a}	11.9 ± 0.4^{b}	94 ± 3^{b}	282 ± 25^{ab}
p-value	0.0334	0.1524	0.0032	0.0000	0.0451

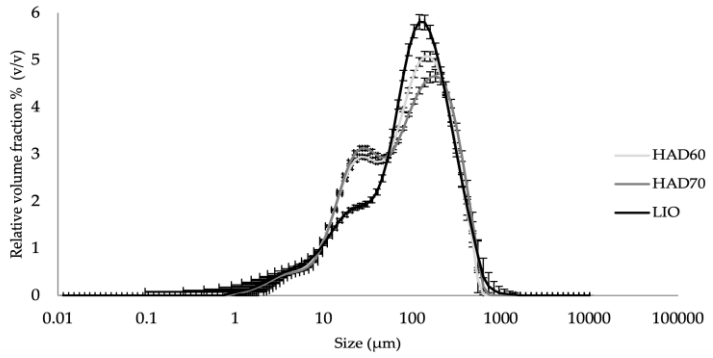

Figure 3.1. Particle size of hot air dried (HAD60: hot air dried at 60 °C; HAD70: hot air dried at 70 °C) and freeze-dried (LYO) almond bagasse powders. Mean ± standard deviation of five repetitions. Different superscripts letters in the same column indicate statistically significant differences with a confidence level of 95%.

Regarding antioxidant properties (Table 3.2), the highest values of antiradical activities were obtained for fresh samples. No significant differences were observed between the different drying methods and temperatures used. The total phenols of the freeze-dried samples were very similar to those of the fresh samples. Freeze-drying occurs at low temperature and in vacuum conditions, which contributes to maintaining bioactive compounds with anti-radical activity such as phenols [40]. In hot air-drying treatments, structural damage and the presence of oxygen at high temperature resulted in higher degradation. However, for the inactivation of enzymes involved in some of the degradation reactions, the difference between 60 and 70 °C could be decisive. In terms of interaction with other molecules, it has been shown that dehydration can increase polyphenolic compounds, despite some degradation, because it can improve extraction and lead to a greater release of these compounds [55]. In almond bagasse, the macronutrient composition, consisting mainly of fibre and fat, could interact with the polyphenols and prevent them from getting released after processing. Comparing results from the DPPH and ABTS methods, the ABTS radical reacted with more antioxidant compounds. The lower reaction time of ABTS radical and its more hydrophilic nature enabled it to react in both organic and aqueous media.

Table 3.2. Total phenols content and antiradical capacity by DPPH and ABTS methods of fresh almond bagasse and, hot air-dried (HAD60: hot air-dried at 60 °C; HAD70: hot air-dried at 70 °C) and freeze-dried (LYO) almond bagasse powders. Mean ± standard deviation of three repetitions. Different superscripts letters for the same determination indicate statistically significant differences with a confidence level of 95%. $_{dm}$, dry matter; GAE, acid gallic equivalent; TE, Trolox equivalent.

	FRESH	HAD60	HAD70	LYO	p-value
Total phenols (mg GAE/g_{dm})	0.59 ± 0.03^{a}	0.291 ± 0.012^{b}	0.33 ± 0.02^{b}	0.5 ± 0.2^{ab}	0.0000
DPPH (mg TE/g_{dm})	0.67 ± 0.06^{a}	0.296 ± 0.007^{b}	0.31 ± 0.03^{b}	0.32 ± 0.05^{b}	0.0154
ABTS (mg TE/g_{dm})	2.9 ± 0.2^{a}	0.96 ± 0.03^{b}	1.03 ± 0.07^{b}	1.121 ± 0.012^{b}	0.0000

The values for total phenols were quite similar to those reported for almond shell, ranging from 0.86 to 1.16 mg GAE/g_{dm} [56], but higher values were found in fresh almonds (2.87 mg GAE/g_{dm}), brazil nuts (2.44 mg GAE/g_{dm}), hazelnuts (6.87 mg GAE/g_{dm}), and pecans (1,81 mg GAE/g_{dm}) [57]. Similar results were obtained in peach (0.51 mg GAE/g_{dm}), fig (0.59 mg GAE/g_{dm}), macadamias (0.46 mg GAE/g_{dm}), and pines (0.32 mg GAE/g_{dm}) [58,59].

3.2. Air Drying Kinetics

Figure 3.2 shows the drying and drying rate curves of thin-layer air-drying of almond bagasse at 60 and 70 °C. The almond bagasse was dried from the initial moisture of 55% to a final value of around 5.5%. The time needed to reduce the water content was 4.5 and 3.7 h at 60 and 70 °C, respectively. As expected, the statistical analysis revealed the significant effect (p-value < 0.05) of air temperature on water content removal during the process. When the air temperature increased, it had a greater capacity to retain water, promoting the drying process. At the same time, the temperature of the bagasse increased significantly, increasing the water diffusivity from the inner layers to the surface [60]. Furthermore, Ling et al. [61] suggested that in pasty products, such as sludge, this temperature increase was linked to a porosity

reduction. Fresh almond bagasse was slightly pasty, so the reduction in porosity could have also contributed to the increase in the drying rate.

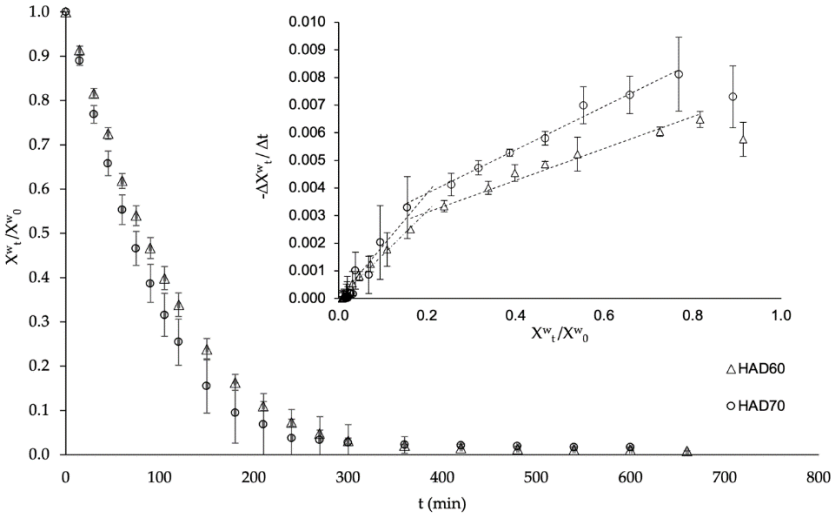

Figure 3.2. Air drying curves of almond bagasse at 60 and 70 °C. In the nested figure, the drying rate curves and the linear fits of the experimental data at 60 and 70 °C have been plotted. HAD: Hot air drying; X^w: water content (g water/g dry matter); 0, t are referring to initial and any other time. Mean values and standard deviation of three repetitions have been represented.

During the first few minutes of the air-drying process, the drying rate increased until it reached the highest value (Figure 3.2). This increase was associated with the progressive heating of the product when it comes into contact with the hot air. The experimental data revealed that this stage, which corresponds to the induction stage, had a duration of 20–30 min, depending on the drying temperature. After the induction period, in high moisture foods, a water-free layer over the entire surface of the food usually results in a constant drying rate [62]. However, the initial moisture content of the fresh bagasse was around 55%, which was low enough that there was no longer a free layer of water. Thus, drying rate curves revealed that the process at the temperature values took place in the falling rate period entirely. Two periods of

declining drying rate were observed; in both cases, the decrease in drying rate was linear with the reduction in moisture ratio Xw/Xw0. Therefore, it could be said that the drying process was controlled by internal water diffusion. In the first stage, when the bagasse had the higher water content, the reduction in velocity was lower than in the second stage when the bagasse was almost dry. This behaviour was largely influenced by the composition and structural characteristics of the bagasse. Considering that the main components of the bagasse do not have a high water-holding capacity (this will be discussed later in the sorption isotherms section), it can be stated that its structural characteristics, in particular its porosity and particle size, determined the facility with which water molecules were removed. Additionally, the extent of compartmentalization associated with the crushing level influences physical interactions that also affect the rate of the process.

Modelling the drying curves and obtaining the kinetic parameters provides information on the mechanisms involved. Furthermore, it makes it possible to control the process by improving energy consumption and subsequently optimize the drying process for greater efficiency and a better quality final product. Numerous models have been used by researchers [63]. These are theoretical, semi-theoretical, and empirical models that usually correlate the moisture ratio with the drying time. Theoretical models provide insights to the mechanisms involved in water loss but offer complex mathematical solutions that are difficult to fit and manage. On the other hand, empirical models provide simple and fast solutions that are effective for practical operation management when the experimental conditions under which they are obtained correspond to the real operating conditions. Semi-theoretical models are the most applied and are generally derived from a direct solution of Fick's second law by assuming some simplifications.

In this study, the experimental data were fitted to an empirical model that establishes a linear correlation between the drying rate and the moisture ratio and to the simplified diffusional model, considering a single term of the serial progression from the integration of Fick's second law (Table 3.3). The simplified diffusional model usually fits well when drying occurs in the falling rate, as this is when the predominant mechanism is the diffusion of water from the innermost layers of the food samples to the surface. In the application of the equation, it was assumed that water diffusion occurred in a single direction and remained constant, the material was isotropic, and the moisture distribution uniform. The external resistance to water transport was negligible compared to the internal resistance and there was no shrinkage or swelling of the food material. The adjustment allowed the calculation of the effective moisture diffusivity (De) as a kinetic parameter to compare the facility with which water diffuses from the inner part of the bagasse to the outer part. The values obtained were 1.97×10^{-9} and 2.18×10^{-9} m^2/s for the temperatures of 60 and 70 °C, respectively. These values are within the range generally given for the moisture diffusion of food materials (10^{-11} to 10^{-6} m^2/s) [64,65].

Saravacos and Maroulis [66] investigated the effect of food properties on the drying kinetics of non-cellular structured food. They established the important effect of food structure and hygroscopicity and reported typical values of effective water diffusivity, varying from 50 to 0.01 × 10−10 m^2/s depending on hygroscopicity. Xiong et al. [67] showed that the effective diffusivity (De) was higher in pregelatinized samples and was found to be much higher through porous puffed pasta than regular pasta. Ruimin et al. [68] found that the total drying time of sludge particles with a diameter of 10 mm is not much different from that of particles with a diameter of 6 mm, while the total drying time of particles with a diameter of 18 mm increases significantly.

Table 3.3. Air drying kinetics of almond bagasse at 60 and 70 °C. $\frac{X^w-X^w_\infty}{X^w_c-X^w_\infty}$: Dimensionless moisture ratio, $\frac{Xw}{Xw_0}$: Moisture ratio, $\frac{\Delta Xw}{\Delta t}$: Drying rate, De: Effective water diffusivity, L: Half-thickness of bagasse thin layer, t: time, R^2: Correlation coefficient, RMSE: Root mean square error, MRE: Mean relative error.

	Model equation	60 °C	70 °C
Linear empirical model	Stage 1 $\frac{\Delta X^w}{\Delta t}=k_1\frac{X^w}{X^w_0}+k_2$	$\frac{X^w}{X^w_0}\in\lfloor 0.816, 0.2\rfloor$	$\frac{X^w}{X^w_0}\in\lfloor 0.769, 0.18\rfloor$
	k_1	0.006	0.008
	k_2	0.002	0.002
	R^2	0.971	0.983
	RMSE	$6.40\cdot10^{-4}$	$9.36\cdot10^{-5}$
	MRE	0.049	0.031
	Stage 2 $\frac{\Delta X^w}{\Delta t}=k'_1\frac{X^w}{X^w_0}$	$\frac{X^w}{X^w_0}\in\lfloor 0.2, 0.02\rfloor$	$\frac{X^w}{X^w_0}\in\lfloor 0.18, 0.022\rfloor$
	k'_1	0.016	0.019
	R^2	0.995	0.921
	RMSE	$1.26\cdot10^{-5}$	$1.05\cdot10^{-4}$
	MRE	0.194	0.207
Difusional model	$\frac{X^w-X^w_\infty}{X^w_c-X^w_\infty}=\frac{8}{\pi^2}e^{\left(\frac{D\cdot\pi^2\cdot t}{4\cdot L^2}\right)}$	$\frac{X^w}{X^w_0}\in\lfloor 0.816, 0.02\rfloor$	$\frac{X^w}{X^w_0}\in\lfloor 0.769, 0.022\rfloor$
	De (m^2/h)	$7.11\cdot10^{-6}$	$7.88\cdot10^{-6}$
	R^2	0.993	0.983
	RMSE	0.039	0.033
	MRE	0.331	0.310

The goodness of the fit was determined by the correlation coefficient (R^2), the root mean square error (RMSE), and the mean relative error (MRE). It is generally accepted that an R^2 value higher than 0.93 and an MRE lower than 0.1 are good fits. Although, the MRE of the fit to the simplified diffusional Fick's model is too high, the correlation coefficient is good and could be accepted as an acceptable approach. In the case of the linear empirical model, the fit was more accurate.

3.3. Sorption Isotherms

Figure 3.3 shows the moisture sorption isotherms at 20 °C of hot air-dried almond bagasse powder at 60 °C (HAD60), at 70 °C (HAD70), and the freeze-dried one (LYO).

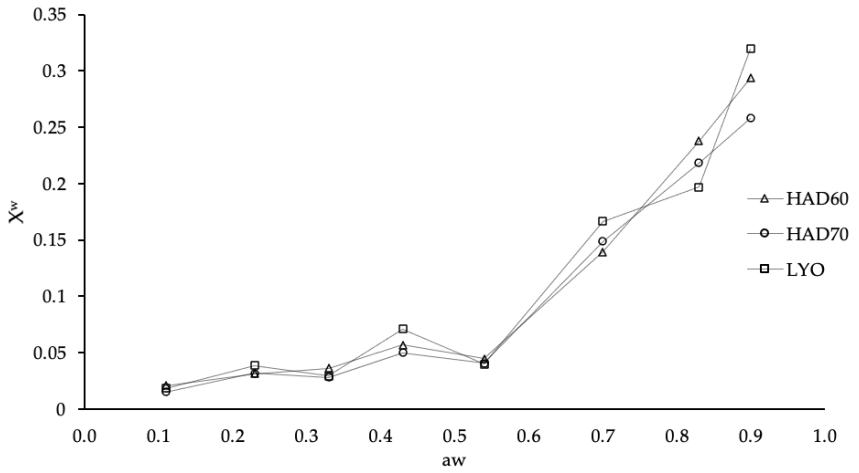

Figure 3.3. Sorption isotherms of hot air-dried (HAD60: hot air-dried at 60 °C; HAD70: hot air-dried at 70 °C) and freeze-dried (LYO) almond bagasse powders at 20 °C. X^w: water content (g water/g_{dm}).

It can be observed that at rather low moisture values (~0.3 g water/g dry matter), water activity values of 0.9 are reached. The isotherm is very close to the x-axis, which indicates that the product has a very low water binding capacity, possibly influenced to a large extent by its fat content. Two practically linear sections can be identified; a rather flat first section for water activities equal to or less than 0.54, and a second section with a positive slope for water activities equal to or greater than 0.54 This results in a type III isotherm, which is quite common in non-porous foods. This shape appears when the net heat of sorption is small (specifically with a BET C value of less than 2). A small net heat of sorption indicates that the interactions

between the water and the other components are weak and more linked to physical than chemical phenomena [69].

When comparing the isotherm with that obtained for raw almond powder [70], the typical plateau at very low aw has disappeared. This plateau is associated with high water adsorption by complex molecules with many active points, such as carbohydrates or soluble proteins. These have been extracted during the production process of vegetable almond drink and are no longer present in the bagasse.

In powdered products, physical and chemical sorption phenomena are largely conditioned by the macromolecular structure of the product as well as by its chemical composition and the physical state of its components [71]. Regarding the macromolecular structure, in all cases, a powder with large and slightly caked particles was obtained, which greatly limits the adsorption phenomena. Considering the composition, the fat content, which is hydrophobic in nature, together with insoluble long-chain carbohydrates (insoluble fibre constituted mainly of cellulose and lignin) is high, and the water adsorption capacity is low. Furthermore, the drying processes applied, such as hot air drying and freeze-drying, may have induced phase transitions aimed at the crystallization of some molecules, resulting in very small or zero stoichiometric hydration contents.

3.4. Conclusions

Hot air drying and freeze-drying were found to be suitable processes for obtaining a plant-based powder from the bagasse resulting from the production of vegetable almond drink. In all cases, a nutritious powder was obtained with low water binding capacity and therefore good properties for packaging and storage. However, due to its high fat content, it is worth studying its stability when stored. No clear trend was

observed for the effect of the drying method (hot air or freeze-drying) on total phenolic content, antiradical capacity, physico-chemical properties, or interaction with water or oil. However, faster kinetics at 70 °C resulted in higher industrial productivity. Freeze-drying resulted in a powder with a more homogeneous particle size distribution and better oil-interaction properties, especially with higher emulsifying activity and stability. It would be the most recommended process to obtain a powder with emulsifying properties.

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ARTÍCULO 2: Shelf life of almond bagasse powders as influenced by dehydration method and storing conditions.

ABSTRACT

Bagasse resulting from the extraction of plant-based beverages represents a promising by-product with potential for use as a functional ingredient. To facilitate its utilisation, the stability of this material can be achieved through dehydration processes such as hot air drying or freeze-drying. Nevertheless, owing to its high fat content, almond bagasse is prone to lipid oxidation, which could result in undesirable quality. Therefore, the objective of this work was to assess the impact of dehydration (by hot air drying at 60 and 70 °C and by freeze-drying) and storage (at room temperature and in accelerated conditions) on the functional quality and stability of almond bagasse powder. Throughout the dehydration process, it was observed that antioxidant compounds were preserved without significant differences among dehydration treatments. These compounds exhibited an increase at the end of the storage period, particularly noticeable in the samples treated with hot air. The acidity and peroxide index were increased by the extended storage period, although they did not reach critical values.

Keywords: nuts; almond bagasse; air drying; freeze drying; valorization; peroxide index; plant-based by-products.

1. Introduction

Almond (*Prunus dulcis*) belongs to the subfamily Prunoideae, family Rosaceae (Ahmad, 2010). The kernel is a highly nutritious plant-based food and due to the numerous health benefits associated with their regular consumption, it is gaining popularity as a healthy food (Ahmad, 2010). Almonds are referred to as "the king of nuts" because are amongst the most widely consumed nuts globally (R. G. M. De Souza et al., 2017; Kamil & Chen, 2012). They are commercially cultivated in various countries, such as the United States, Spain, Australia, Morocco, Iran, Turkey, and Chile. Globally, its production has increased from 1.03 million metric tons in 2014-2015 to 1.48 million metric tons in 2019-2020 (*Almond Production Worldwide 2024 | Statista*, n.d.). Specifically, almond plantations are extensively spread across the Mediterranean region, encompassing a total area of 2,162,263 hectares. Spain holds the largest area dedicated to almond cultivation, reaching 744,470 hectares (*FAOSTAT*, n.d.).

Numerous studies have been conducted on the composition of almond kernel, revealing their high content in nutrients such as fatty acids, lipids, amino acids, proteins, carbohydrates, dietary fibres and bioactive compounds such as phytosterols, polyphenols, minerals and vitamins (Tomishima et al., 2022; Yada et al., 2011). The total fat content ranges from 32% to 66% and it is mostly monounsaturated fat, providing them with remarkable oxidative stability (Fernandes et al., 2017). Oleic acid predominates as the primary fatty acid, constituting 50% to 70% of the total fatty acids. Linoleic, palmitic, and stearic acids are present at levels from 10% to 26%, 5% to 9%, and 1.5% to 4%, respectively. The concentrations of linolenic and myristic acids are exceedingly low, less than 0.1% (Martínez et al., 2013). Almonds contain approximately 10-29% of protein. While they are considered

relatively high in protein compared to other nuts, they do not provide a complete range of essential amino acids, thus excluding them as a source of high biological value protein (T. S. P. de Souza, Dias, Oliveira, et al., 2020). Additionally, almonds contain about 14 to 26.6% of dietary fibre, primarily located in the walls of the almond kernel. The dietary fibre found in almonds has been reported to have prebiotic effects on the gut microbiota (Liu et al., 2014). Almonds offer a variety of bioactive compounds, with phenolic compounds being most notable. Approximately 130 phenolic compounds have been identified in almonds to date (Bolling et al., 2010). The key phenolic compounds include chlorogenic acid (9.5 mg/100 g), catechin (11.04 mg/100 g), epicatechin (12.47 mg/100 g), and isorhamnetin-3-O-rutinoside (48.5 mg/100 g) (Bolling, 2017).

In recent years, plant-based beverages have gained immense popularity, either due to the increase in lactose intolerance or for ideological reasons. Among them, almond-based vegetable drinks stand out (Lorente et al., 2023). The production process is straightforward, involving a solid-liquid extraction using water as a solvent. The resulting solid residue, known as pulp or bagasse, is frequently discarded or repurposed as animal feed (Bartkiene et al., 2020b). Nonetheless, this residue contains a significant amount of nutritional compounds such as lipids, proteins, and bioactive components. The intriguing composition of the bagasse resulting from the production of almond-based vegetable drinks makes it a potential suitable substrate for producing functional ingredients beneficial for the food industry (Duarte, Betoret, et al., 2023). Stabilizing bagasse through various dehydration methods, such as hot air drying or freeze-drying, is a valuable strategy to improve its durability and preserve its bioactive components (Ramírez-Pulido et al., 2021). As a result, it is possible to obtain functional powdered ingredients with interest for the food industry (Duarte, Betoret, et al., 2023). The quality and shelf life of almond bagasse

powder is affected by environmental factors such as humidity, temperature, light, and oxygen content in the storage atmosphere due to their high content of mono- and di-unsaturated fatty acids. While these are exogenous factors that promote oxidation processes, there are also endogenous factors, such as oxidative enzymes found in these natural foods, such as lipoxigenase (LOX); their activation usually occurs when plant tissue is disrupted [13]. Thus, the most common deteriorative reactions include oxidation with increasing acidity values and peroxide index.

Therefore, this study aims to assess the impact of dehydration (by hot air drying at 60 and 70 °C, and freeze-drying) and storage at room temperature and accelerated conditions on the functional quality and stability of almond bagasse powder. Specifically, it seeks to explore the effects of dehydration conditions, temperature and storage time on humidity, water activity, higroscopicity, optical properties, acidity, peroxide index, antiradical capacity, polyphenol profile and microbiological stability of almond bagasse powder.

2. Materials and Method

2.1. Process for obtaining almond bagasse and almond bagasse powder.

Naturally peeled almonds were purchased from a local supermarket and soaked with tap water at a ratio of 1/9 (w/w) for 18 hours. The soaked almonds were ground at 10,000 rpm for 20 seconds using a domestic food processor (Thermomix®, Vorwerk, Spain), and subsequently filtered through a 500 µm stainless steel sieve to recovery the almond bagasse for further characterization.

For obtaining a powder, the almond bagasse was evenly distributed on a plastic grid with a nominal opening of 2 mm and air-dried until the water activity (aw) was

below 0.3. A convective dryer (Pol-eko Aparatura, Katowice, Poland) with cross air flow at 60 or 70 °C was used to obtain the air-dried bagasse (HAD60 and HAD70). The lyophilised bagasse samples (LYO) were obtained from almond bagasse previously frozen at -40 °C for 24 h and after freeze dried in a lyophiliser (Telstar, Lioalta-g). The dried almond bagasse, HAD and LYO, was ground in a food processor (Thermomix®, Vorwerk, Spain) in two stages: first at 4,000 rpm for 20 s at 5 s intervals, then at 10,000 rpm for 20 s at 5 s intervals to obtain a coarse-grained powder.

2.2. Evaluation of storage stability of the almond bagasse powder

The almond bagasse powders dried at 60, 70 °C and lyophilised were packed in heat-sealed, aluminum-laminated polyethylene bags (200 g) and stored at room temperature (27 ± 2 °C, 50-75% RH) and at accelerated conditions (38 ± 2 °C, 90 ± 2% RH) for 180 days. Water activity, moisture, optical properties, hygroscopicity, acidity, total phenols, antiradical activity, peroxides index were determined every 15 days and specific phenolic compounds were determined every 90 days. Microbiological analyses (moulds and yeasts and total mesophiles) were performed monthly. Each analysis was performed in triplicate.

2.3. Physico-chemical properties

The water activity of almond bagasse powders (HAD60, HAD70 and LYO) was determined with a dew point hygrometer (DECAGÓN Aqualab 4TE) at 20 °C.

The moisture content was determined following the official method in dried fruits established by the AOAC (AOAC 934.06, 1996).

Hygroscopicity was evaluated according to the method described by (Cai & Corke, 2000); 0.5 g of each sample was weight into a glass crucible and placed into a closed

chamber with saturated sodium sulphate solution (Na_2SO_4) for 7 days at 25 °C. After 7 days the weight gain was measured.

The CIE*L*a*b* coordinates were determined with a spectrocolourimeter (MINOLTA, CM-3600D, Japan), using the standard light source D65, the standard 10° observer and the surface reflectance spectra from 400 to 700 nm. Hue (hab), chroma (Cab) and colour difference (ΔE) were calculated by equations 3.6, 3.7 and 3.8.

$$h_{ab} = \text{arctg}\left(\frac{b}{a}\right) \qquad (3.6)$$

$$C_{ab} = \sqrt{a^2 + b^2} \qquad (3.7)$$

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \qquad (3.8)$$

Where a*, b* and L* represent the colour coordinates in the CIE colour space, L stands for lightness, a* is the red-green component and b* the yellow-blue component. hab, Cab and ΔE refer to the hue, chroma and colour difference of each powder after storage for a period of time t compared to powder before storage, respectively.

2.4. Acidity and peroxide value

The standardized method proposed by Rani et al. (2018) was employed to determine the acidity. Following the pH measurement, the sample was neutralized with 0.5 M NaOH until it reached a pH of 8.20. Results were expressed as g oleic acid/ 100 g dry matter.

The peroxide index was determined in accordance with the official AOAC method (AOAC, 2000) for the peroxides value of oils and fats. 0.5 g of sample were weighed

and solubilized in an aqueous solution containing 18 mL of acetic acid and 12 mL of chloroform. After that, 0.5 mL of a saturated solution of KI and 30 mL of distilled water were added and titrated with a 0.01M $Na_2S_2O_3$ solution.

2.5. Determination of phenolic compounds and antiradical capacity

For the extraction of antioxidants, 1 g of sample was weighed and mixed with 10 mL of a mixture of methanol and water in a ratio of 80:20 (v/v) as solvent solution. After 1 h of shaking (Intelli-Mixer RM-2M), the sample was centrifuged (Selecta, "Medrifriger BL-S") at 10,000 rpm and 20 °C for 5 min. Determinations were carried out on the supernatant, hereafter called the extract.

2.5.1. Total phenol content

The determination of total phenols was carried out according to the Wolfe et al. (Wolfe et al., 2003) description. In a spectrophotometric cuvette, 0.125 mL of Folin-Ciocalteu reagent (Sigma-Aldrich), 0.125 mL of extract and 0.5 mL of double distilled water were added and allowed to react for 6 min. After this time, 1.25 mL of 7% (w/v) sodium carbonate (w/v) solution, and 1 mL distilled water were added. A reagent blank was made; the extract was replaced with double distilled water and allowed to react for 90 min. The absorbance was then measured at 765 nm in a spectrophotometer (Thermo Scientific, Helios Zeta U/Vis). The results obtained were compared with a standard curve for gallic acid (purity ≥ 98%) and expressed as mg of gallic acid equivalents/g of dry matter (mg GAE/g dm).

2.5.2. Antiradical capacity by DPPH and ABTS methods

The antioxidant capacity was determined following the DPPH procedure described by Stratil et al. (Stratil et al., 2006) and Kuskoski et al. (Kuskoski et al., 2005) with some variations. 0.1 mL of the extract and 2.9 mL of the methanol-DPPH (39.4 mg/mL) solution were mixed, and the absorbance was measured at 517 nm in a spectrophotometer (Thermo Scientific, Helios Zeta U/Vis). The results were compared with a Trolox calibration curve ($C_{14}H_{18}O_4$, purity ≥ 7%, Sigma-Aldrich) for the concentration range between 0 and 500 mg/L. The results were expressed as milligram Trolox equivalents per gram of dry matter (mg TE/g dm).

Antioxidant activity was also assessed by the ABTS radical method (2,2'-azino-bis-(3-etil benzotiazolin-6-sulphonic acid)) following the methodology proposed by Re et al. (Re et al., 1999). A solution of the acid (7 mM, purity ≥ 99%) was prepared with potassium persulphate (2.45 mM, purity 99.99%) in distilled water and incubated in the dark at room temperature for 16 h. It was then diluted with methanol to an absorbance of 1.0 ± 0.02 at 734 nm. As blank, the sample was replaced by double distilled water. The absorbance was measured after 0, 3- and 7-min reaction time in a spectrophotometer (Thermo Scientific, Helios Zeta UV/Vis) at a wavelength of 734 nm. The results were expressed as mg of trolox equivalent/g of dry matter (mg TE/g dm).

2.5.3. Phenolic compounds by HPLC analysis

Specific polyphenolic compounds were determined following the method proposed by Caprioli et al. (2018) and Giusti et al. (2019). For acid hydrolysis, 2.5 g of sample was weighed and 7.5 mL of solvent (70:30 v/v) ethanol and double distilled water) was added, pH was adjusted to 4 with HCl and left for 2 h in an ultrasonic bath

at room temperature, after that the samples were centrifuged at 8000 g for 15 min. This extraction process was performed twice on the solid sample. The supernatants were filtered with 20 µm polytetrafluoroethylene (PTFE) and the resulting extract analysed by HPLC.

Samples extracts were analysed using a 1200 series rapid resolution HPLC coupled to a series diode array detector (Agilent, Palo Alto, CA, USA). Phenolic compounds were separated on a Brisa-LC 5 column (Tanleque-Alberto et al., 2020). Mobile phase A was 1% formic acid and mobile phase B was acetonitrile. The gradients were: 0 min 10% B, 25 min 60% B, 26 min 80% B holding up to 30 min, 35 min 10% B holding up to 40 min. The working conditions were an injection volume of 0.5 mL/min and a flow rate of 10 µL at 30 °C. The phenolic compounds were detected at different chromatographic retention times determined by reference standards. The wavelengths of each compound were: vanillin, 250 nm; 260 nm for rutin, 4-hydroxybenzoic acid and quercetin 3-glucoside; 280 nm for chlorogenic acid and epicatechin; 320 nm for sinapic acid, ferulic acid, p-coumaric acid and 7-glucoside of apigenin. The compounds were quantified by calibration curves and the results were expressed as mg/100 dm.

2.6. Microbiological analyses

A 10 g sample of each almond bagasse powder was aseptically transferred into sterile stomachers bags. Samples were mixed with 90 mL of sterile peptone solution (Scharlab, Barcelona, Spain) and homogenized for 2 min. Serial dilutions were made for plate inoculation in 9 mL of sterile peptone. Plate count agar (Scharlab, Barcelona, Spain) was used for total plate count and potato dextrose agar (Scharlab, Barcelona, Spain) for yeast and mold count. Total mesophiles plate count, yeasts and moulds were incubated at 37 ± 2 °C for 24 h and at 27 ± 2 °C for 72 h, respectively.

2.7. Statistical Analysis

The results underwent statistical analysis using Statgraphics software (Centurion XVI.I, Statpoint Technologies, Inc., Warrenton, VA, USA) at a 95% confidence level (p-value ≤ 0.05). The normality of the data was assessed using the Shapiro-Wilk test ($p > 0.05$). Following this, an analysis of variance (ANOVA) was conducted. Each treatment transformation was replicated in three separate experiments, with three replicates in each experiment. Fisher's LSD test was utilised to identify any significant differences (p-value < 0.05) among groups.

3. Results and discussion

Table 3.4 shows physicochemical properties of almond bagasse after air drying at 60 °C and 70 °C and freeze-drying. The difference in water activity (aw) among the hot air-dried samples at different temperatures and the freeze-dried ones (samples HAD60, HAD70, and LYO) is striking. This may be due to the structural fracture produced during the freezing and sublimation stages. This structural rupture facilitates the removal of more bound water, allowing the water activity to be reduced to a greater extent. In hot air-dried samples, the higher drying temperature causes phase transitions and a compact and deformed structure which makes water removal more difficult. In all cases water activity was reduced to below 0.3, which is the recommended threshold for ensuring microbiological and physicochemical stability in powdered products such as milk powder or instant coffee (Inglett et al., 2015; Vesterlund et al., 2012). Nevertheless, as the storage period progressed, there was an observed increase in water activity for all samples (Table 3.5 and 3.6). This increase was most pronounced for samples subjected to accelerated storage, with the values for samples HAD60 AC, HADO70 AC, and LYO AC increasing by 3, 2, and 8 times their initial values, respectively. The higher porosity associated with the

structural damage suffered by the freeze-dried samples justifies their higher water uptake capacity. However, while a substantial increase in water activity was noted at the end of the storage period, it did not reach critical levels in any case.

Table 3.4. Water activity, humidity, hygroscopicity, optical properties, peroxide index, acidity, microbial counts and specific phenolic compounds of air dried and lyophilized almond bagasse (HAD60: hot air dried at 60 °C; HAD70: hot air dried at 70 °C; LYO: lyophilized). Mean ± standard deviation of three repetitions. Different superscripts letters in the same line indicate statistically significant differences with a confidence level of 95%. dm, dry matter; aw, water activity; X_w, water content; n.d, not detected.

	HAD60	**HAD70**	**LYO**
a_w	0.13 ± 0.03^b	0.23 ± 0.01^c	0.05 ± 0.01^a
X_w (g/ g_{dm})	0.009 ± 0.009^a	0.007 ± 0.001^a	0.0011 ± 0.0009^a
Hygroscopicity (g_w/ g)	0.116 ± 0.004^a	0.20 ± 0.01^b	0.224 ± 0.007^c
Peroxide (m_{eq}/ kg_{dm})	3.3 ± 0.2^b	3.4 ± 0.2^b	1.9 ± 0.1^a
Acidity (g/ $100g_{dm}$)	0.619 ± 0.008^b	0.622 ± 0.001^b	0.58 ± 0.01^a
Mesophiles (log UFC/ g_{dm})	n.d	n.d	n.d
Mould and yeast (log UFC/ g_{dm})	n.d	n.d	n.d
Phenolic compounds (mg/ 100 g_{dm})			
4-Hydroxibezoic acid	60.0 ± 0.9^a	70.7 ± 0.9^c	84.5 ± 1.4^b
Epicatechin	458 ± 17^a	498 ± 7^b	585.0 ± 0.9^c
Vanillin	94.6 ± 0.9^a	128.4 ± 0.9^c	98.7 ± 1.4^b
Rutin	206.4 ± 0.9^c	195.2 ± 0.2^b	151.6 ± 0.9^a
Quercetin 3-glucoside	63.0 ± 0.9^a	66.1 ± 0.9^b	112.2 ± 0.9^c
p-Coumaric acid	39.0 ± 0.9^a	39.4 ± 0.9^a	51.1 ± 0.9^b
Sinapic acid	61.6 ± 0.4^a	63.9 ± 0.4^b	120.5 ± 0.5^c
Ferulic acid	63.1 ± 0.4^a	65.0 ± 0.4^b	107.8 ± 0.5^c
Apigenin-7-glucoside	60 ± 11^a	65 ± 10^a	36 ± 16^a
Clorogenic acid	78.2 ± 0.9^a	81.6 ± 0.9^b	87.4 ± 0.9^c
Sum of specific phenols	1181 ± 12^a	1272 ± 1^c	1435 ± 9^b
Colour			
L	58.1 ± 0.5^b	51.3 ± 0.5^a	61.86 ± 0.09^c
a*	7.15 ± 0.06^a	8.1 ± 0.2^b	8.0 ± 0.6^b
b*	15.27 ± 0.05^a	19.3 ± 0.4^c	16.5 ± 0.4^b
C	64.93 ± 0.02^a	67.2 ± 0.4^c	65.1 ± 0.5^b

RESULTADOS Y DISCUSIÓN

The moisture content in the samples was low, and there were no significant differences among the various drying methods employed. However, the rate of water absorption in the samples during storage was noted to be higher under accelerated conditions. This absorption significantly increased, reaching values up to 3, 4, and 25 times higher than their initial levels for the HAD60 AC, HAD70 AC, and LYO AC samples, respectively. However, even though there was moisture absorption by the end of the storage period (180 days), the moisture content remained below 10% so is still considered low.

Hygroscopicity is the capacity of a material or powder to absorb moisture and come into equilibrium with relative humidity of the environment. As per Arlindo et al. (Medeiros Arlindo et al., 2007), the hygroscopic properties of specific foods largely depend on their chemical composition and storage conditions, including relative humidity. Significant differences were observed among air dried samples at 60 °C and 70 °C and lyophilized ones, being higher the values in the last. As a consequence, the lyophilized almond powders showed an increased humidity and water activity after storage as it has been remarked previously. The hygroscopic values can be considered low-medium, as a material can be considered non-hygroscopic if less than 20% is observed (Callahan et al., 2016). This could elucidate the increased moisture absorption under accelerated conditions. Comparable results have been documented for guava pulp powder and melon powder, both stored under normal and accelerated conditions (Breda et al., 2012; S. L. Tan et al., 2021).

The acidity of food is attributed to the presence of organic acids that serve as substrates for respiration, and variations in this parameter can impact the quality characteristics of the food (Papadimitriou et al., 2016). The acidity (Figure 3.4) showed significant differences ($p < 0.05$) between air-dried samples and freeze-dried

ones and, also, significant differences ($p < 0.05$) were observed among samples HAD60, HAD70 and LYO in terms of storage at different conditions. The samples stored for 180 days at room temperature presented values ranging from 0.56 to 0.97 g/ 100 g dry matter, with the lowest acidity observed in the freeze-dried samples and the highest in the samples dried at 70 °C. The samples subjected to accelerated storage for 180 days exhibited a significant increase compared to those stored at room temperature; there was a 73%, 61%, and 25% increase for samples HAD60 AC, HAD70 AC, and LYO AC, respectively. According to (Morrison (1963), the increase in acidity in stored grains and flours over an extended period is due to an elevation in the concentration of free fatty acids, which leads to greater food deterioration. As stated by (Fargerson., 1969; Angelo et al., 1979), other potential factors contributing to the increased acidity in samples stored at higher temperatures include the interaction of the amino group in amino acids, short-chain peptides, and proteins, resulting in the release of carboxylic ends while generating acidic by-products from advanced Maillard reactions. Considering that almond bagasse powder is rich in fatty acids and proteins, the observed increases can be attributed to both phenomena, especially in the hot air-dried samples. However, even with accelerated storage, the values obtained after 180 days of storage are acceptable, taking into account that in foods such as olive oil, values of up to 1 g oleic acid/100 g can be obtained without affecting quality (CEE, 1991).

The peroxide values for air-dried samples at 60 and 70 °C and freeze-dried ones ranged from 1.9 to 3.4 meq O_2/kg dm (Figure 3.4). Significant differences ($p < 0.05$) were observed between treatments, as the hot air drying process is more aggressive on terms of oxidative reactions due to the presence of oxygen and the high temperature resulting in a higher peroxide index (Nader et al., 2021). Samples stored for 180 days showed peroxide values ranging from 10 to 12.8 meq O_2/kg dry matter

for samples stored at room temperature and from 14.7 to 23 meq O_2/kg dry matter for samples subjected to an accelerated storage. The peroxide index values were higher in accelerated storage for the different drying methods. Similar results were found by El Bernoussi et al. (2020) in almond oils subjected to accelerated storage for 4 weeks, with peroxide indices ranging from 2.4 to 24.6 O_2/kg. As the temperature increases, oleic, linoleic, and linolenic acids produce hydroperoxides. These substances decompose, resulting in the creation of a broad spectrum of secondary oxidation products. Hydroperoxides are primarily formed and decomposed during the initial stages. Nevertheless, as the storage period progresses, the rate of formation substantially rises, leading to a significant elevation in the overall concentration of hydroperoxides. Consequently, this heightened concentration contributes to increased oxidation. The oxidation rate escalates exponentially with temperature. Additionally, there is also an interaction between oxygen and temperature, as higher temperatures lead to decreased reduced oxygen availability. Similar trends were observed in dried hazelnuts stored for 21 months, showing an increase of up to twice their initial value (Turan & Karaosmanoğlu, 2019). Likewise, comparable results were obtained in pistachios stored for 15 months, recording values of 14.9 meq O_2/kg dry matter (Maskan & Karataş, 1999) and in milk powder stored for 9 months where values of 11.3 meq O_2/kg dry matter were obtained.

Regarding microbiological stability, the dehydration process significantly reduces the available water for microbial growth, resulting in null values for the final microbial load. Although no significant differences were noted in cell counts between drying methods ($p \leq 0.05$), there was a gradual increase in these values as the storage period extended. Following 180 days of storage, samples subjected to an accelerated storage process exhibited higher levels of mesophiles, molds, and yeasts.

Nevertheless, it is essential to highlight that these values did not surpass critical levels, so all samples met the minimum safety requirements (BOE, 2011).

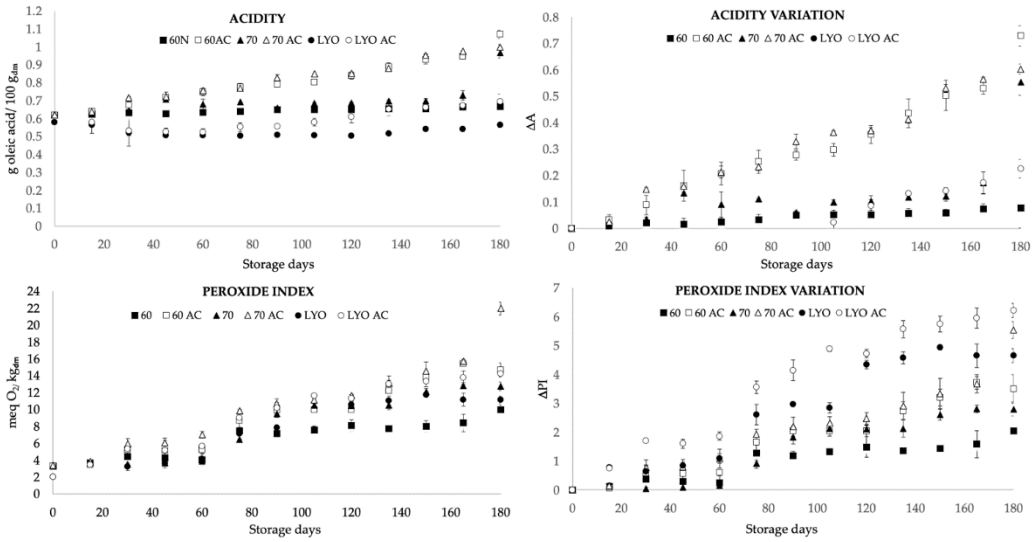

Figure 3.4. Acidity, peroxide index, acidity variation (ΔA) and peroxide index variation (ΔPI) for air-dried at 60 °C (HAD60) and 70 °C (HAD70) and freeze-dried (LYO) almond bagasse powders during 180 days of storage at room temperature and in accelerate conditions (AC). Variation has been calculated as the difference between the value at the time t minus the value at time 0 relative to the value at time 0. Mean ± standard deviation has been represented.

The dehydration treatment significantly ($p \leq 0.05$) affected the content of specific polyphenols ($p \leq 0.05$), although the effect depended on the chemical nature of the considered component (see Table 3.4). The samples subjected to hot air drying (HAD60 and HAD70) showed reduced polyphenol content compared to the freeze-dried samples, except for rutin, which demonstrated values of 206.4 ± 0.9 and 195.2 ± 0.2 for samples HAD60 and HAD70 respectively, and for apigenin-7-glucoside with values of 60 ± 11 and 65 ± 10 in samples HAD60 and HAD70 respectively. Once more,

the moderate temperature and the absence of oxygen during freeze-drying minimised potential for degradation. In addition, Maillard reaction products generated during air drying treatment can chelate flavour compounds, transition metal ions and polyphenols, reducing the possibilities of being adequately quantified (Shakoor et al., 2022). However, freezing and sublimation did lead to the degradation of the cellular structure, promoting the formation of a more porous matrix structure. This circumstance could, over time, facilitate the loss of some phenolic compounds as in the case of rutin and apigenin-7-glucoside. Understanding the reasons why temperature (in the case of air-drying) or structural degradation (in the case of freeze-drying) has a greater influence on the degradation of certain components would require further work taking into account the release of polyphenols in each treatment; the kinetics of degradation reactions associated with each compound; and other compounds interactions. The temperature during the hot air drying process had a significant impact on the total phenols content ($p \leq 0.05$). In addition, in most cases drying at 60 °C led to lower phenol content compared to drying at 70 °C. These temperature differences could play a decisive role in the inactivation of enzymes involved in specific degradation reactions. Storage also had a significant impact on the specific polyphenol contents (see Tables 3.5 and 3.6). An increase was observed in all samples, but samples subjected to accelerated storage showed an even greater increase. Specifically, for 4-hydroxybenzoic acid, epicatechin and vanillin, increases of 8%, 63% and 59% respectively were recorded for the HAD60 AC samples; 29%, 90% and 126% respectively for the HAD70 AC samples; and 14%, 13% and 12% respectively for the LYO AC samples. Storage might induce physical transformations in components like cellulose or lignin, thereby facilitating access to the phenolic profile for extraction. Another potential explanation is the continuation of polyphenol synthesis after almonds are harvested, a phenomenon observed in

some food items. For instance, in peanuts, there was a 42% increase in polyphenols after 24 months of storage (Sreeramulu, 1983). A similar trend was noted in sprouted groundnuts, which displayed an elevated content of total phenols. Likewise, a comparable pattern emerged in almond shells stored at 23 °C for 9 months, indicating an 84% rise in catechin, a 50% increase in epicatechin, and an 18-fold increase in hydroxybenzoic acid compared to the initial levels (Bolling et al., 2010).

The rise in total phenol content after 180 days of storage manifested prominently in the total polyphenolic profile, particularly in the samples subjected to accelerated storage. Notably, HAD60 AC, HAD70 AC, and LYO AC exhibited increases of 25%, 52%, and 7%, respectively. A substantial elevation was observed in the HAD70 AC samples, potentially attributed to the utilization of high temperatures. These elevated temperatures might have induced the generation of Maillard reaction byproducts, renowned for their exceptional antioxidant capacity (Nooshkam et al., 2019). Nevertheless, previous studies indicated that storing nuts for extended periods results in a significant decrease in the levels of polyphenols and antioxidant compounds. For instance, the storage of peanuts at temperatures of 20 °C and 35 °C for up to 4 months revealed a 35% reduction in total polyphenols compared to the initial levels (Talcott et al., 2005). Similarly, a 42% decrease in green tea catechins was noted following storage at 20 °C for 4 months (Friedman et al., 2009). Also, raw almonds stored in darkness for up to 24 months lost between 83 to 90% of their vitamin E content (Friedman et al., 2009).

Table 3.5. Water activity, humidity, hygroscopicity, optical properties, peroxide index, acidity, microbial counts and phenolic compounds for air dried and lyophilized almond bagasse (HAD60: hot air dried at 60 °C; HAD70: hot air dried at 70 °C; LYO: lyophilized) after 90 days of storage at room temperature and in accelerated conditions (AC). Mean ± standard deviation of three repetitions. Different superscripts letters in the same line indicate statistically significant differences with a confidence level of 95%. dm, dry matter; aw, water activity; X_w, water content.

	HAD60	HAD70	LYO	HAD60 AC	HAD70 AC	LYO AC
a_w	0.245 ± 0.001^b	0.274 ± 0.002^c	0.10 ± 0.01^a	0.341 ± 0.001^b	0.41 ± 0.02^c	0.30 ± 0.02^a
X_w (g/g_{dm})	0.013 ± 0.01^b	0.0137 ± 0.0002^b	0.0030 ± 0.0001^a	0.017 ± 0.001^a	0.026 ± 0.004^v	0.0183 ± 0.0005^b
Hygroscopicity (g_w/ g)	0.195 ± 0.006^a	0.220 ± 0.004^b	0.22 ± 0.02^b	0.202 ± 0.009^a	0.208 ± 0.005^a	0.208 ± 0.006^a
Peroxide (m_{eq}/g_{dm})	7.14 ± 0.08^a	9.5 ± 0.4^c	7.9 ± 0.1^b	9.92 ± 0.02^a	10.7 ± 0.5^b	10.2 ± 0.7^b
Acidity (g/ 100 g_{dm})	0.65 ± 0.01^b	0.68 ± 0.01^b	0.52 ± 0.03^a	0.79 ± 0.01^c	0660 ± 0.006^b	0.579 ± 0.004^a
Mesophiles (log UFC/ g_{dm})	1 ± 0^b	0^a	1 ± 0^b	1.5 ± 0.7^b	0^a	2 ± 0^c
Mould and yeast (log UFC/ g_{dm})	1 ± 0^b	0^a	1 ± 0^b	1.5 ± 0.7^a	1.5 ± 0.7^a	1.5 ± 0.7^a
Phenolic compounds (mg/ 100 g_{dm})						
4-Hydroxibezoic acid	69.7 ± 0.5^b	72.1 ± 0.1^c	28.7 ± 0.3^a	66.7 ± 0.6^b	72.6 ± 0.6^c	34.3 ± 0.5^a
Epicatechin	460.6 ± 0.1^a	773.7 ± 33^b	701.0 ± 38^b	441 ± 86^a	829.9 ± 17^b	697.6 ± 21^b
Vanillin	256.8 ± 4^b	310.9 ± 1^c	87.3 ± 0.9^a	229.8 ± 2^b	294.5 ± 3^c	79.0 ± 0.9^a
Rutin	154.7 ± 0.3^a	153.1 ± 0.8^a	256.3 ± 3^b	162.9 ± 0.5^b	146.7 ± 0.03^a	242.2 ± 1.1^c
Quercetin 3-glucoside	63.0 ± 0.04^b	51.9 ± 0.2^a	106.9 ± 1.2^c	61.4 ± 0.8^b	51.5 ± 1.2^a	82.1 ± 1^c
p-Coumaric acid	36.6 ± 1.1^a	52.9 ± 0.3^c	39.9 ± 0.04^b	59.3 ± 1.3^c	53.1 ± 1.7^b	39.6 ± 0.8^a
Sinapic acid	78.0 ± 0.7^b	87.0 ± 0.9^c	69.9 ± 0.5^a	80.4 ± 0.6^c	85.2 ± 0.3^b	72.6 ± 2^a
Ferulic acid	77.3 ± 0.8^b	83.2 ± 0.8^c	70.9 ± 1^a	79.0 ± 0.8^b	82.5 ± 1^c	72.0 ± 0.6^a
Apigenin-7-glucoside	77 ± 1^a	69.2 ± 0.6^a	102 ± 6^b	75.5 ± 0.1^b	69.6 ± 0.3^a	100.8 ± 0.8^c
Clorogenic acid	82.2 ± 3^a	88.7 ± 1^a	98.6 ± 1^b	85.8 ± 1^a	102.40 ± 0.01^b	99.7 ± 2^b
Sum of specific phenols	1356.4 ± 0.4^a	1743 ± 29^c	1562 ± 25^b	1342 ± 4^a	1788 ± 8^c	1520 ± 3^b
Colour						
L	58.6 ± 0.2^a	59.3 ± 0.2^a	61.7 ± 0.6^b	57.22 ± 0.12^a	58.7 ± 0.2^b	72.94 ± 0.01^c
a*	6.99 ± 0.14^{ab}	6.80 ± 0.04^a	7.06 ± 0.14^b	7.33 ± 0.02^c	7.30 ± 0.01^b	5.29 ± 0.01^a
b*	15.15 ± 0.49^a	15.72 ± 0.09^{ab}	15.78 ± 0.03^b	15.86 ± 0.12^b	16.55 ± 0.09^c	14.67 ± 0.01^a
C	16.69 ± 0.51^a	17.13 ± 0.07^{ab}	17.29 ± 0.03^b	17.10 ± 0.01^b	18.09 ± 0.08^c	15.60 ± 0.01^a
ΔE	0.6 ± 0.4^a	1.3 ± 0.6^a	1.3 ± 0.7^a	1.15 ± 0.1^a	6.6 ± 0.5^b	3.19 ± 0.04^c

Table 3.6. Water activity, humidity, hygroscopicity, optical properties, peroxide index, acidity, microbial counts and specific phenolic compounds for air dried and lyophilized almond bagasse (HAD60: hot air dried at 60 °C; HAD70: hot air dried at 70 °C; LYO: lyophilized) after 180 days of storage at room temperature and under accelerated conditions (AC). Mean ± standard deviation of three repetitions. Different superscripts letters in the same line indicate statistically significant differences with a confidence level of 95%. dm, dry matter; aw, water activity; X_w, water content.

	HAD60	HAD70	LYO	HAD60 AC	HAD70 AC	LYO AC
a_w	0.30 ± 0.03^{ab}	0.34 ± 0.03^{b}	0.280 ± 0.002^{a}	0.431 ± 0.002^{a}	0.484 ± 0.002^{b}	0.432 ± 0.004^{a}
X_w (g/g_{dm})	0.021 ± 0.001^{b}	0.024 ± 0.001^{c}	0.0154 ± 0.0004^{a}	0.0314 ± 0.0004^{b}	0.032 ± 0.002^{b}	0.0292 ± 0.0005^{a}
Hygroscopicity (g_w/ g)	0.168 ± 0.006^{a}	0.214 ± 0.005^{b}	0.213 ± 0.005^{b}	0.179 ± 0.01^{a}	0.220 ± 0.013^{b}	0.223 ± 0.006^{b}
Peroxide (m_{eq}/g_{dm})	10.0 ± 0.6^{a}	12.8 ± 0.5^{c}	11.2 ± 0.4^{b}	14.7 ± 0.9^{a}	23 ± 2^{b}	14.3 ± 0.3^{a}
Acidity (g/ 100g $_{dm}$)	0.666 ± 0.004^{b}	0.97 ± 0.03^{c}	0.56 ± 0.01^{a}	1.07 ± 0.02^{c}	1.00 ± 0.01^{b}	0.734 ± 0.009^{a}
Mesophiles (log UFC/ g_{dm})	2.5 ± 0.7^{b}	1 ± 0^{a}	2.5 ± 0.7^{b}	3 ± 0^{c}	1.5 ± 0.7^{a}	3 ± 0^{c}
Mould and yeast (log UFC/ g_{dm})	2.5 ± 0.7^{c}	1 ± 0^{a}	2 ± 0^{b}	4 ± 0^{b}	3 ± 0^{a}	4 ± 1^{c}
Phenolic compounds (mg/ 100 g_{dm})						
4-Hydroxibezoic acid	80.8 ± 1.3^{b}	75.6 ± 0.2^{a}	89 ± 2^{c}	64.6 ± 0.4^{a}	91.0 ± 0.4^{b}	96 ± 4^{c}
Epicatechin	463 ± 7^{a}	747 ± 6^{c}	607 ± 4^{b}	747.5 ± 6.1^{b}	945.6 ± 24.7^{c}	659.1 ± 0.9^{a}
Vanillin	263 ± 6^{b}	322 ± 2^{c}	113 ± 1^{a}	150 ± 2^{b}	290.3 ± 0.5^{c}	110.7 ± 1.2^{a}
Rutin	143.6 ± 0.9^{b}	145 ± 3^{b}	75 ± 2^{a}	111 ± 2.0^{a}	173.4 ± 0.4^{b}	226.1 ± 0.3^{c}
Quercetin 3-glucoside	68 ± 2^{b}	61.4 ± 0.4^{a}	81 ± 2^{c}	53.2 ± 1.1^{a}	56.4 ± 0.6^{a}	76.9 ± 1.4^{b}
p-Coumaric acid	55 ± 2^{c}	38 ± 1^{a}	48.5 ± 0.2	53.4 ± 0.4^{c}	46.3 ± 0.7^{a}	49.1 ± 0.2^{b}
Sinapic acid	80 ± 2^{a}	91.1 ± 1.5^{b}	76.2 ± 0.4^{a}	71.4 ± 1.2^{a}	84 ± 2^{b}	74 ± 2^{a}
Ferulic acid	79 ± 2^{b}	86.9 ± 0.8^{c}	74.0 ± 1.2^{a}	71.5 ± 0.1^{a}	84.7 ± 1.1^{b}	72.9 ± 0.3^{a}
Apigenin-7-glucoside	80.6 ± 0.1^{b}	75 ± 2^{a}	81 ± 6^{b}	68.3 ± 1.1^{a}	71.6 ± 1.4^{a}	79 ± 8^{b}
Clorogenic acid	75 ± 2^{a}	87.7 ± 0.9^{b}	88.0 ± 1.4^{b}	83.2 ± 0.6^{a}	95.1 ± 1.2^{c}	91.0 ± 0.4^{b}
Sum of specific phenols	1388 ± 10^{b}	1923 ± 1^{c}	1336 ± 1^{a}	1475 ± 9^{a}	1938 ± 4^{c}	1541 ± 2^{b}
Colour						
L	57.883 ± 0.004^{b}	54.5 ± 0.3^{a}	60.20 ± 0.01^{c}	56.593 ± 0.006^{a}	56.73 ± 0.01^{b}	61.587 ± 0.002^{c}
a*	6.85 ± 0.01^{a}	8.98 ± 0.13^{b}	6.958 ± 0.002^{a}	9.00 ± 0.01^{b}	7.0 ± 0.6^{a}	7.664 ± 0.002^{a}
b*	14.84 ± 0.01^{a}	18.1 ± 0.4^{a}	15.25 ± 0.01^{a}	19.09 ± 0.02^{c}	14.93 ± 0.01^{a}	16.643 ± 0.004^{b}
C	65.22 ± 0.01^{a}	63.6 ± 0.3^{c}	65.47 ± 0.01^{b}	64.74 ± 0.02^{c}	65.972 ± 0.003^{a}	65.274 ± 0.003^{b}
ΔE	0.7 ± 0.3^{a}	5.0 ± 0.4^{c}	2.3 ± 0.4^{b}	4.5 ± 0.2^{b}	7.1 ± 0.6^{c}	3.56 ± 0.01^{a}

Table 3.7. Total phenols content and antiradical capacity by DPPH and ABTS methods for hot air-dried (HAD60: hot air-dried at 60 °C; HAD70: hot air-dried at 70 °C) and freeze-dried (LYO) almond bagasse powders during 180 days of storage at room temperature and in accelerate conditions (AC). Mean ± standard deviation of three repetitions. Different superscripts letters for the same determination indicate statistically significant differences with a confidence level of 95%. dm, dry matter; GAE, acid gallic equivalents; TE, Trolox equivalent.

	Days	HAD60	HAD70	LYO	HAD60 AC	HAD70 AC	LYO AC
DPPH (mgTE/g $_{dm}$)	0	0.20 ± 0.02^{a}	0.23 ± 0.01^{a}	0.24 ± 0.007^{a}	0.20 ± 0.02^{a}	0.23 ± 0.01^{b}	0.319 ± 0.007^{c}
	60	0.201 ± 0.013^{a}	0.218 ± 0.02^{a}	0.22 ± 0.02^{a}	0.2372 ± 0.0013^{b}	0.21 ± 0.01^{a}	0.333 ± 0.009^{c}
	90	0.391 ± 0.006^{b}	0.37 ± 0.01^{ab}	0.36 ± 0.01^{a}	0.37 ± 0.01^{b}	0.34 ± 0.01^{a}	0.38 ± 0.01^{b}
	180	0.31 ± 0.02^{a}	0.307 ± 0.014^{a}	0.344 ± 0.009^{b}	0.27 ± 0.01^{b}	0.213 ± 0.009^{a}	0.404 ± 0.007^{c}
ABTS (mgTE/g $_{dm}$)	0	1.04 ± 0.02^{b}	1.092 ± 0.014^{b}	0.56 ± 0.05^{a}	1.04 ± 0.02^{b}	1.092 ± 0.014^{b}	0.56 ± 0.05^{a}
	60	0.73 ± 0.02^{b}	0.788 ± 0.012^{c}	0.53 ± 0.02^{a}	0.75 ± 0.08^{ab}	0.84 ± 0.04^{b}	0.65 ± 0.04^{a}
	90	0.88 ± 0.05^{b}	0.95 ± 0.04^{c}	0.44 ± 0.01^{a}	0.86 ± 0.03^{b}	0.91 ± 0.04^{c}	0.52 ± 0.02^{a}
	180	0.79 ± 0.05^{b}	0.99 ± 0.03^{c}	0.55 ± 0.02^{a}	1.07 ± 0.01^{c}	0.813 ± 0.003^{b}	0.60 ± 0.02^{a}
Phenols (mg GAE/g $_{dms}$)	0	0.31 ± 0.02^{a}	0.355 ± 0.006^{b}	0.313 ± 0.007^{a}	0.31 ± 0.02^{a}	0.355 ± 0.006^{b}	0.313 ± 0.007^{a}
	60	0.39 ± 0.02^{b}	0.535 ± 0.015^{c}	0.291 ± 0.004^{a}	0.47 ± 0.02^{b}	0.54 ± 0.02^{b}	0.3 ± 0.4^{a}
	90	0.323 ± 0.002^{a}	0.37 ± 0.01^{b}	0.32 ± 0.01^{a}	0.44 ± 0.03^{b}	0.43 ± 0.02^{b}	0.34 ± 0.01^{a}
	180	0.443 ± 0.011^{b}	0.51 ± 0.02^{c}	0.242 ± 0.006^{a}	0.63 ± 0.06^{c}	0.552 ± 0.011^{b}	0.248 ± 0.013^{a}

Table 3.7 shows the values of antiradical capacity determined by the DPPH and ABTS methods and the content of total phenols. Regarding to the effect of dehydration method, it was observed that the antiradical capacity measured by DPPH showed no significant differences between the samples. However, concerning the antiradical capacity assessed by ABTS, the samples dried by hot air exhibited higher values compared to the freeze-dried ones. This could be attributed to the products formed through Maillard reactions induced by temperatures applied in the hot air-drying process. These reactions involve amino acids and generate lipid oxidation products. According to Nooshkam et al. (2019), the resulting products from Maillard reactions possess excellent antiradical capacities. The differences observed in the results obtained from the DPPH and ABTS methods stemmed from their distinct sensitivities to antiradical compounds. The ABTS radical was found to interact with a greater number of antioxidant compounds. Its shorter reaction time and more hydrophilic nature enable it to react in both organic and aqueous environments (Ozgen et al., 2006). The total phenols exhibited similar behaviour, with hot air-dried samples showing higher values than the freeze-dried ones. However, notably, the samples dried at 70 °C displayed even higher values. This could be pivotal in terms of other degradative enzymes inactivation.

Contrarily, the changes of antiradical capacity during storage of hot air-dried and freeze-dried almond bagasse powders, show a gradual increase in all dehydrated samples over time. Higher values were noted in the samples subjected to an accelerated storage. With the DPPH method, there was an increase of 0.085 mg TE/g of dry matter for the LYO AC samples while the HAD60 samples showed an increase of 0.07 mg TE/g dry matter. Regarding the ABTS method, an increase was observed in the HAD60 AC and LYO AC samples of 0.03 and 0.04 mg TE/g dry matter,

respectively. Similar findings were reported by Del Caro et al. (2004) who observed an augmentation in antiradical capacity over time in minimally processed citrus segments and juices. This phenomenon can be attributed to the ongoing formation of new compounds with antioxidant properties, such as Maillard reaction products, which persist even after extended storage periods. Throughout the storage period, a noticeable increase in total phenols was observed, particularly evident in samples undergoing accelerated storage conditions. Specifically, samples HAD60 AC and HAD70 AC at 6 months of storage exhibited an increase of 0.32% and 55%, respectively. These findings align with those reported by Bolling et al. (2010) who similarly observed an augmentation in phenolic compounds within almond skins under comparable storage conditions. However, the LYO AC sample experienced a 21% loss in total phenols. This phenomenon could be related to the freeze-drying process which, despite occurring at low temperatures and under vacuum conditions, produces an important structural rupture that favours the diffusion of oxygen and the oxidation of phenolic compounds during storage. In summary, for specific polyphenolic compounds, temperature and oxygen have a significant degradative effect during processing and storage, causing a significant decrease in the content of all the components analysed, except for rutin, apigenin-7-glucoside and vanillin. However, the effects are more pronounced with hot air at 60 °C than at 70 °C. On the other hand, results follow a very different evolution when referring to non-specific measures such as antiradical activity or total phenolic content. In these cases, it seems that lipid oxidation and Maillard reactions, which occur mainly during hot air drying (and to a greater extent at 70°C) and continue during storage, generating reaction products which have a high antiradical capacity and phenolic nature (Shakoor et al., 2022).

The different dehydration methods provided almond bagasse powders with significantly different colour coordinates (see Table 3.4). This difference arises from browning and oxidation reactions, resulting in more reddish and yellowish hues in the samples air dried at 70 °C (HAD70), as indicated by higher values in the a* and b* coordinates. In contrast, the freeze-dried samples displayed greater lightness (L) due to the reduced browning caused by the moderate temperatures and the absence of oxygen during the process. At the end of the 180-day storage period, it was observed that samples HA60 and LYO, practically did not exhibit significant differences in terms of colour variation compared to the initial samples (see ΔE in Table 3.6). However, in the case of sample HAD70, a slight increase in colour variation was noted (5.0 ± 0.4), and this trend was consistent in HAD70 AC. Notably, it should be highlighted that colour differences are imperceptible to the human eye when ΔE is less than 1 but become visibly apparent when the ΔE value exceeds 3 (Bodart et al., 2008). Additionally, it can be observed that the colour variation during storage (Table 3.5 and 3.6), have slightly increased in the samples stored at room temperature and that more intense changes have occurred in the samples stored under accelerated conditions. These results are consistent with the fact that Maillard reactions continue during storage and become more intense as the temperature increases.

4. Conclusions

Both hot air drying and freeze drying have provided a powder with water activity and hygroscopicity values that ensure its stability for three months even under accelerated storage conditions. Moreover, the increase in peroxide value and acidity during storage at ambient temperature is sufficiently moderate to ensure good quality of the final product in all cases.

RESULTADOS Y DISCUSIÓN

The effect of storage on the content of specific polyphenols or the antiradical capacity is mainly determined by the structural changes caused by the dehydration operation and the formation of compounds in reactions such as the Maillard reactions in hot air drying. In any case, understanding the reasons why temperature or structural changes has a greater influence on the degradation of certain components or the generation of others would require further work taking into account the release of polyphenols in each treatment; the kinetics of degradation reactions associated with each compound; and other compounds interactions.

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ARTÍCULO 3: Suitability of almond bagasse powder as a wheat flour substitute in biscuit formulation.

ABSTRACT

Almond bagasse, a by-product derived from the production of almond vegetable drink, contains antioxidants, fibre, protein, and a high fat content, presenting itself as a potential functional ingredient for the food industry. This study aimed to assess the powder derived from almond bagasse as a suitable alternative in the formulation of bakery goods. Various formulations substituting wheat flour with almond bagasse powder, obtained by air drying or freeze-drying at 10%, 15%, and 25%, were analysed in terms of technological, and rheological properties. Furthermore, the physical and antioxidant attributes of biscuits with superior nutritional and functional value produced using these blends were examined. The results revealed significant changes in oil retention capacity, stability and emulsifying activity, influenced by both the level of wheat flour replacement and the drying method used to obtain the almond bagasse powder. On the other hand, the lack of starch and the high concentration of fat and insoluble fibre in the almond bagasse powder determined the viscoelastic behaviour of the hydrated blends; as the percentage of substitution with the almond bagasse powder increased, the final viscosity decreased. It is also worth noting that the use of these blends for biscuit preparation resulted in a final product with a higher content of antioxidant components.

Keywords: almond bagasse powder; air drying; freeze drying; blends; antioxidants.

1. Introduction

Bakery products such as bread, biscuits and cakes, among others, are widely consumed in the human diet and in Western countries are usually made mainly from refined wheat flour (Martins et al., 2017). Unfortunately, the wheat proteins can trigger digestive or allergic problems in some individuals (Demirkesen & Ozkaya, 2022). Moreover, refined flour have lost nutrients during processing, resulting in products with lower content of minerals, vitamins, and fibre and consequently with reduced nutrient content, higher caloric value, and an increased glycemic index (Parenti et al., 2020). Additionally, the excessive production of these items has led to a substantial expansion in wheat cultivation for flour, potentially causing adverse environmental impact (Pourmehdi & Kheiralipour, 2020).

On the other hand, there is currently a growing interest in valorising and reintegrating waste from the food industry into the food chain. Significant amounts of waste and by-products are typically generated during the processing of plant-based foods, leading to economic and environmental issues due to the high volumes and associated costs of disposal (Mateos-Aparicio & Matias, 2019). These waste materials may contain high levels of proteins, dietary fibre, essential fatty acids, antioxidant compounds, vitamins and minerals and can be easily stabilised by transformation into powdered products (Difonzo et al., 2022). Therefore, the incorporation of powdered waste as ingredients with high nutritional and/or functional value in the development of new products could lead to healthier processed products. For example, functional powders made from vegetable, legume or nut waste could be beneficial as they would allow the nutritional supplementation of other types of flours with lower nutritional content. In addition, they could contribute to reduce intolerance or allergy problems and to lower the glycemic index,

thus preventing diseases such as celiac disease, type 2 diabetes, coronary heart diseases, or certain cancers (Difonzo et al., 2022; Lu et al., 2013).

Other researchers have conducted studies employing nuts and revalorized by-products as ingredients in the production of bakery products. Aguilar et al. (2015) used powdered tiger nut beverage by-product to make gluten-free bread. Pycia & Ivanišová (2020) enriched wheat bread with ground hazelnuts and walnuts. Aguilar et al. (2015) explored the use of chickpea and tiger nut flour as alternatives to emulsifiers and shortening in gluten-free bread making. Gaglio et al. (2023) incorporated almond skin powder in traditional semolina sourdough bread production. Owiredu et al. (2014) replaced wheat flour with cashew nut flour in biscuit production. Ramos et al. (2023) employed almond baru flour in cake development. Pop et al. (2020) used walnut cake by-product, derived from oil extraction, in macaron preparation. The incorporation of alternative ingredients to wheat in the baking process involves technological challenges to achieve acceptable quality products. Generally, the substitution of bread-making flours with ingredients derived from by-product valorisation varies between 2% and 20%, with 10% being the most accepted level by consumers due to their organoleptic characteristics (Dhankhar et al., 2019; Ibrahium & Hegazy, 2014; Zhao et al., 2019). It should be noted that the inclusion of gluten-free or starch-free ingredients can influence the elasticity and gas retention capacity in the dough, which would weaken its structure affecting the quality of the final product (Petitot et al., 2010). On the other hand, the substitution percentage of by-products in blends significantly impacts the nutritional and functional properties of bakery products, such as protein, fat content, dietary fibre, vitamins, minerals or antioxidant compounds. Furthermore, it also influences technological and functional properties, including water holding capacity, oil absorption capacity, swelling, gelling and thickening (Mateos-Aparicio & Matias,

2019). Technological and functional properties play a key role in determining the suitability of a blend in the production of bakery products, exerting a significant impact on the texture, structure and overall quality of such products. The flour's ability to interact with water is essential in forming an appropriate dough, as it should be able to absorb and retain water to achieve an elastic and malleable dough (Gómez et al., 2003). In addition, the ability to interact with oil is also important, as it is essential for incorporating fat into the dough, which could affect the softness, moisture, and juiciness of the final product. This interaction can also influence the texture and shelf life of the product (Pareyt et al., 2011).

The almond bagasse powder obtained from the by-product of almond vegetable drink production could be a suitable raw material to replace part of the wheat flour in the production of bakery products. It contains monounsaturated fatty acids and essential amino acids and has good water and oil interaction properties (Duarte et al., 2023). However, it lacks certain amino acids, such as methionine, lysine, and threonine (Cowan et al., 1963). Conversely, wheat flour protein is predominantly comprised of gliadin and glutenin, which encompass a diverse range of amino acids. Nevertheless, it is important to note that these proteins can elicit allergic reactions in individuals sensitive to gluten (Anne Van et al., 2005). The objective of this work was to evaluate the suitability of the powder obtained from almond bagasse as a substitute ingredient in the production of bakery products. With this aim, different formulations replacing wheat flour with almond bagasse powder, obtained by air drying or lyophilisation, at 10%, 15% and 25% were characterised in terms of technological, pasting, and rheological properties. The physical and antioxidant properties of the biscuits with increased nutritional and functional value made with the blends were also determined.

2. Materials and method

2.1. Process for obtaining almond bagasse and almond bagasse powder.

Local supermarket-bought almonds were soaked in tap water at a ratio 1/9 (by weight). Using a household food processor (Thermomix®, Vorwerk, Spain) running at 10,000 rpm for 20 seconds, the soaked almonds were ground into a fine mixture. The resulting grind was later sifted through a stainless-steel sieve with a mesh size of 500 µm. The almond bagasse, left behind on the sieve, was collected for further analysis and processing. It was found that the weight of the collected almond bagasse was approximately 82% of initial almonds weight.

To obtain the dehydrated almond bagasse, the almond bagasse was first evenly distributed on plastic grids with a nominal opening of 2 mm. It was then subjected to a hot air drying in a convective dryer (Pol-eko Aparatura, Katowice, Poland) with a cross-flow of air at a speed of 10 m/s and a temperature of 60 °C for 10 hours, until it reached a water activity (aw) below 0.3. This process resulted in the air-dried almond bagasse (HAD). Additionally, a freeze dryer (Telstar, Lioalta-g) was used to obtain the lyophilized (LYO) product from the almond bagasse previously frozen at -40 °C for 24 hours. Afterward, both dehydrated almond bagasse were ground using a food processor (Thermomix®, Vorwerk, Spain) at 4,000 rpm for 20 seconds with 5-second intervals, and then at 10,000 rpm for 20 seconds with 5-second intervals, resulting in the almond bagasse powder. Lastly, the powder was stored at 20 °C in opaque glass jars to prevent any deterioration and oxidation reactions. The almond bagasse powders (HAD and LYO) were mixed in proportions of 10, 15 and 25% with refined wheat flour to obtain blends, which were characterized.

2.2. Physicochemical analysis of wheat flour and almond bagasse powder blends.

The official procedure from AOAC was employed to determine **moisture** content (AOAC 934.06) and **fat** content (AOAC 991.36) in the blends. For fat content determination a Soxhlet extraction with petroleum ether using a 5 g sample to 90 mL solvent ratio at 290 °C was applied. **The ash** content was determined by subjecting the material to incineration in a muffle furnace at 550 °C, following the AOAC 940.26 protocol. For **bulk density**, the method proposed by Amandikwa et al. (2015) was followed with some modifications. A known sample weight was added to a graduated cylinder, tapped gently to compact the sample, and the occupied volume was me registration. The results were expressed in g/mL.

2.3. Water interaction and emulsifying properties

Water holding capacity (WHC) is the quantity of water that the sample can retain without the need for external pressure. To determine this, 0.2 g of the sample were mixed with 10 mL of distilled water; the mixture was allowed to sit at 25 °C for 18 hours (Robertson et al., 2000); the excess water removed; and the water content of the resulting solid determined. **Water absorption capacity** (WAC) refers to the ability of a sample to absorb and hold water even under external forces, such as centrifugation (Robertson et al., 2000). To determine this, 1 g of the sample was weighed into a graduated conical tube, and 10 mL of distilled water was added, allowing it to stand at 25 °C for 18 hours. Subsequently, the mixture was subjected to centrifugation at 2,000 rpm for 30 minutes. The liquid portion was separated, and the weight of the sedimented residue was measured. The method suggested by Garau et al. (2007) was employed to assess **the oil absorption capacity**. A combination of 0.2 g of the sample and 1.5 g of sunflower oil was prepared and

allowed to stand at 20 °C for an entire night. Following this, the mixture underwent centrifugation at 3,416 rpm for 5 min, during which the supernatant was extracted using a Pasteur pipette, and the weight of the residue was measured. The evaluation of oil absorption capacity was based on the rise in sample weight, and the outcomes were presented in terms of g of absorbed oil per g of initial sample (g_o/g_s). The assessment of **emulsifying activity** was carried out following the approach outlined by Yasumatsu et al. (2014). To perform the procedure, a solution consisting of 2% (w/v) sample and water was prepared. Subsequently, 7 mL of this solution was mixed with 7 mL of sunflower oil, and the mixture was homogenized for 5 minutes using a vortex at a speed of 2,400 rpm. Finally, the mixture was subjected to centrifugation at 10,000 rpm for 5 minutes, and the volume of the resulting emulsion was determined by calculating the ratio between the emulsion volume and the total fluid volume. The assessment of **emulsifying stability** was conducted following the procedure proposed by Yasumatsu et al. (2014). To accomplish this, a solution containing 2% (w/v) sample and water was prepared. Subsequently, 7 mL of this solution was combined with 7 mL of sunflower oil and homogenized for 5 minutes using a vortex at a speed of 2,400 rpm. Following that, it was subjected to heating at 80 °C for 30 minutes, allowed to cool, and then centrifuged at 2,000 rpm for 5 minutes. Emulsifying stability was determined by calculating the ratio between the emulsion volume and the total fluid volume.

2.4. Pasting properties

The viscometric profile of the blends was obtained following the procedure described by Harasym et al. (2020) in accordance with ICC Standard method 162 and using a Rapid Visco Analyser (RVA-4500, Perkin Elmer, USA). A quantity of 3.5 g of the sample was transferred to an RVA container, and distilled water (as the solvent) was

added to achieve a total weight of 28.5 g. Each hydrated blend was kept at 50 °C for 1 minute to reach equilibrium. Subsequently, the temperature was gradually increased to 95 °C at a rate of 5 °C per minute, held at 95 °C for 5 minutes, then cooled to 50 °C at a rate of 5 °C per minute, and finally maintained at 50 °C for the last 4 minutes. The stirring was initiated at 960 rpm for the first 10 seconds and then maintained at a constant 160 rpm for the rest of the analysis. Each sample underwent duplicate analysis. The TCW3 software (Perkin Elmer, United Kingdom) was used to calculate the parameters of peak viscosity (PV), trough viscosity (TV), final viscosity (FV), setback (ST = FV-TV), gelatinization temperature, and time to peak viscosity.

2.5. Rheological measurements

For rheological determinations, dynamic oscillatory tests were performed on hydrated blends (3.5 g blend/ 25 g water). An Anton Paar MC102 rheometer (Anton Paar, Stuttgart, Germany) was used, employing serrated parallel plates (40 mm in diameter) made of steel, with a 1 mm gap, at a controlled temperature of 25°C using a KNX2002 thermal controller. The samples were placed onto the plate, excess removed, and allowed to equilibrate for 5 minutes before each test. Viscoelastic behaviour was assessed through the storage modulus (G') and loss modulus (G'') with a frequency sweep ranging from 10 to 1 Hz, within the linear viscoelastic region and under a constant stress of 1 Pa. All rheological tests were conducted in triplicate.

2.6. Texture analysis.

The hydrated blend was deposited into cylinders with a diameter of 2 mm and refrigerated at 4 °C for 12 hours until a compact gel formed. The texture of the gels was determined in triplicate using an AXIS texture analyzer (Axis, Gdansk, Poland) equipped with FMAXIS software. A double compression test of texture profile

analysis (TPA) was conducted. The gels underwent a 50% deformation test at a speed of 1 mm/s with 30-second intervals between the first and second compression. The results were expressed as maximum force (N) for both compressions.

2.7. Preparation of dough and biscuits.

For the biscuit dough preparation, 100 g of blend was mixed with 30 g of vegetable shortening and rubbed until it was uniform. Subsequently, 25.5 g of water containing 20 g of white sugar and 1 g of salt was added. The dough was kneaded gently on a rolling board until it reached a uniform thickness (5 mm), and then cut using a round cutter with a diameter of 45 mm.

The dough pieces were baked in greased molds at 180 °C for 15 minutes. The baked biscuits were cooled for 20 minutes and stored in an airtight container for subsequent analysis.

2.8. Determination of physical properties in biscuits.

The thickness (T) and diameter (D) of batches containing 10 biscuits each were measured using a vernier caliper. To calculate the relative weight (RW), relative diameter (RD), relative thickness (RT), specific volume (VE) and relative specific volume (REV), equations 3.9, 3.10, 3.11, 3.12, 3.13 respectively. The dispersion ratio was calculated as D/T. The colour meter (MINOLTA, CM-3600D, Japan) was used to determine the CIE*L*a*b* coordinates, considering the standard light source D65, the 10° standard observer, and the surface reflectance spectra ranging from 400 to 700 nm. The chroma (Cab) and colour differences (ΔE) between the wheat flour biscuits and those containing almond bagasse powder were calculated using equations 3.14 and 3.15, respectively.

$$\mathrm{RW} = \frac{\text{weight}}{\text{control weight}} \qquad (3.9)$$

$$\mathrm{RD} = \frac{\text{diameter}}{\text{control diameter}} \qquad (3.10)$$

$$\mathrm{RT} = \frac{\text{thickness}}{\text{control thickness}} \qquad (3.11)$$

$$\mathrm{EV} = \frac{\pi \cdot \text{diameter}^2 \cdot \text{thickness}}{4 \cdot \text{weight}} \qquad (3.12)$$

$$\mathrm{REV} = \frac{\mathrm{EV}}{\text{control EV}} \qquad (3.13)$$

$$\mathrm{C_{ab}} = \sqrt{a^2 + b^2} \qquad (3.14)$$

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (3.15)$$

Where control is refered to the wheat flour.

2.9. Antiradical capacity and total phenols content in biscuits

To extract phenols and other constituents with antiradical capacity, an 80:20 (v/v) mixture of methanol and water was prepared and used as a solvent in a ratio of 1 g of sample per 100 mL solvent. After 1 hour of magnetic stirring, the mixture was centrifugated (Selecta, "Medrifriger BL-S") at 10,000 rpm for 5 minutes at 20 °C. The analyses were carried out on the resulting supernatant, referred to as the extract.

2.9.1. Antiradical capacity by DPPH and ABTS methods

The determination of antiradical capacity was conducted according to the DPPH method outlined by Stratil et al. (2006) with certain modifications. A mixture of 0.1

mL of the extract and 2.9 mL of methanol-DPPH solution (0.394 of DPPH reagent/mL methanol) was prepared, and the absorbance was measured at 517 nm in a spectrophotometer (Thermo Scientific, Helios Zeta U/Vis). The outcomes were presented as milligrams of trolox equivalent per gram of dry matter (mg TE/g dm), utilizing the Trolox calibration line ($C_{14}H_{18}O_4$, purity ≥ 7%, Sigma-Aldrich) as the reference standard antioxidant, across a concentration range from 0 to 500 mg/L.

The evaluation of antioxidant activity was also carried out by the ABTS radical method (2,20-azobis-3-ethyl benzothiazolin-6-sulfonic acid) (Re et al., 1999). A solution containing the ABTS radical (7 mM) and potassium persulfate (2.45 mM) in distilled water was prepared and allowed to incubate in darkness at room temperature overnight. Following this incubation period, a dilution with methanol was performed to achieve an absorbance of 0.7 ± 0.02 at 734 nm. Subsequently, the reaction was initiated in a spectrophotometer cuvette by combining 0.1 mL of the extract with 2.9 mL of the ABTS solution. For comparison, a blank sample was prepared by replacing the extract with distilled water. Absorbance was measured after 0, 3, and 7 minutes of reaction at a wavelength of 734 nm using a spectrophotometer (Thermo Scientific, Helios Zeta UV/Vis). The results were expressed as mg of trolox equivalent/g of dry matter (mg TE/g dm), utilizing the Trolox calibration line ($C_{14}H_{18}O_4$, purity ≥ 7%, Sigma-Aldrich) as the reference standard antioxidant, across a concentration range from 0 to 500 mg/L.

2.9.2. Total Phenol Content

The quantification of total phenols was carried out using the Folin–Ciocalteu colorimetric method (Wolfe et al., 2003). In a spectrophotometer cuvette, 0.125 mL of the extract, 0.125 mL of the Folin–Ciocalteu reagent (Sigma-Aldrich, Darmstadt, Germany), and 0.5 mL of distilled water were sequentially added and allowed to

react for 6 minutes. Afterthat, 1.25 mL of a 7% (w/v) sodium carbonate solution and 1 mL of distilled water were added. A comparative reference was prepared replacing the sample by distilled water and left to react for 90 minutes. Ultimately, the absorbance was measured at 765 nm using a spectrophotometer (Thermo Scientific, Helios Zeta U/Vis). The results were correlated with a standard gallic acid curve (purity ≥ 98%, Sigma-Aldrich) and expressed as milligrams of gallic acid equivalents per gram of dry matter (mg GAE/g dm).

3. Results and discussion

3.1. Proximal composition and interaction properties with water and oil of wheat flour and almond bagasse powders.

Table 3.8 shows proximal composition and water and oil interaction properties of wheat flour and almond bagasse powders dehydrated by hot air at 60 °C and lyophilized. Regarding moisture, the dehydration process has significantly reduced the available water content in almond bagasse dried using either hot air or freeze-drying methods. Unlike these samples, wheat flour maintains a higher moisture level, as it was commercial flour with any additional treatment. Nevertheless, the moisture level remains below critical levels, which is advantageous for its preservation. Considering the fat and protein content the almond bagasse powders exhibits a significant fat content (25%). Almond fat composition consist of mono- and polyunsaturated fatty acids, such as oleic, linoleic, palmitic, stearic, and palmitoleic acids (Sathe et al., 2008). Adequate consumption of these fatty acids offers several health benefits, including cholesterol reduction and a lowered risk of cardiovascular disease (Kamil & Chen, 2012). Lipids represent a minor fraction in wheat flour compared to its other primary nutritional components. The amount of protein found in both almond bagasse powder and wheat flour falls within a similar range,

approximately between 0.16 and 0.12 g of protein/g respectively. Similar findings have been observed in other products, like oat bran (0.17 g protein per gram) (Nedeljković et al., 2017) or soybean residue (0.15 g protein per gram) (Lu et al., 2013). Despite this commonality in protein content, almond bagasse powder and wheat flour differ significantly in fibre and fat content which has an impact in technological properties as we shall see below. It is worth noting the absence of starch in almond bagasse powders, which undoubtedly determines the technological properties of the mixtures and the most recommended applications for them. Starch is fundamental for the structure and texture of bakery products. The absence of starch, when replacing part of the wheat flour with almond bagasse powder, can impact dough elasticity, gas retention, and result in a denser texture. Moreover, almond bagasse powder, with higher fat content, may affect the consistency and structure of the final product, leading to notable alterations in the quality of the baked final product.

Table 3.8. Proximal composition and technological properties of wheat flour and almond bagasse powders dehydrated by hot air at 60 °C (HAD60) and lyophilised (LYO). [1]Duarte et al., 2023, [2]Hager et al., 2012. dm, dry matter; X_w, water content.

	Wheat flour	**HAD60[1]**	**LYO[1]**
Xw (g/g_{dm})	0.108 ± 0.003	0.014 ± 0.002	0.02 ± 0.08
Fat (g/g_{dm})	0.01 ± 0.02	0.252 ± 0.002	0.250 ± 0.006
Ashes (g/g_{dm})	0.0203 ± 0.0002	0.031 ± 0.007	0.030 ± 0.012
Protein (g/ g_{dm})	0.12 ± 1.07[2]	0.16 ± 0.04	0.165 ± 0.008
Fibre Van Soest (g/g_{dm})	0.034[2]	0.45 ± 0.02	0.50 ± 0.03
Cellulose and lignine (g/g_{dm})	0.012[2]	0.20 ± 0.05	0.21 ± 0.02
Hemicellulose (g/g_{dm})	0.021[2]	0.260 ± 0.014	0.295 ± 0.002
Total starch (g/100g)	68.06[2]	-	-
Water holding capacity (gw/g_{dm})	0.56 ± 0.04	2.9 ± 0.5	8.4 ± 1.8
Water absorption capacity (gw/g_{dm})	0.71 ± 0.01	4.5 ± 0.2	5.91 ± 0.08
Oil absorption ability (go/gs)	0.144 ± 0.004	2.3 ± 0.5	4.2 ± 006
Emulsifying stability (%)	-	19 ± 2	34 ± 2
Emulsifying activity (%)	-	20 ± 2	59 ± 2

3.2. Functional properties of the blends prepared replacing wheat flour with hot air dried almond bagasse.

Technological properties, such as water holding capacity (WHC) and water absorption capacity (WAC), are primarily influenced by particle size, starch and fibre content, the amount of fat and the type of proteins. Table 3.8 shows higher values for almond bagasse powders when compared to wheat flour. This could be attributed to the more soluble fibre (some of hemicelullose in this case), which demonstrates a high capacity to retain water and expand. Conversely, insoluble fibre also possesses the ability to retain and absorb water within its fibrous matrix, albeit to a lesser degree. However, the freeze-dried powder demonstrates higher values than the hot air-dried powder, attributable to the particle size distribution (Duarte et al., 2023). According to Bai et al. (2021), as particle size increases, the ability to absorb and retain water also increases mainly due to the posibility of water molecules to traverse the larger gaps between particles. To date, there remains a dearth of substantial studies on the interaction properties with oil. Nonetheless, according to Devnani et al. (2021), almond protein isolate demonstrates foaming and emulsifying properties that could be akin to those found in soy protein isolate (Sze-Tao & Sathe, 2000). Meanwhile, wheat flour plays a vital role in the creation of bakery items owing to its gluten content. This constituent provides elasticity and structure to the dough, enabling optimal retention of the gas generated by the yeast and resulting in fluffier texture.

Table 3.9 includes the results for water, fat and ashes content; bulk density; water and oil interaction properties of the blends prepared replacing wheat flour by hot air dried or lyophilized almond bagasse in the range from 10 to 25%. Regarding moisture, it was significantly affected ($p \leq 0.05$) by the percentage of substitution. As

the percentage of substitution increases, the moisture content decreases. This is due to the inherent low moisture content of almond bagasse powder. A similar trend was observed in biscuits formulated with different substitution levels using Moringa leaf powder (Giuberti et al., 2021). On the other hand, the same trend was observed in relation to the final fat content. Since almond bagasse contains around 25% fat, this means that the higher the percentage of substitution, the higher the amount of fat in the mixture. Regarding ash content, no significant differences were observed. However, concerning bulk density, notable differences ($p \leq 0.05$) were detected in the substitution percentage. Higher values were recorded in HAD60-10% and LYO-10%. It could be attributed to the substantial amount of fat in almond bagasse. Similar results were documented in wheat flour blends with yam powder for biscuit manufacturing (Amandikwa et al., 2015).

Table 3.9. Water, fat and ashes content, bulk density and water and oil interaction properties of the blends prepared replacing wheat flour by hot air dried almond bagasse in the 10% (HAD60-10%), in the 15% (HAD60-15%) or in the 25% (HAD60-25%); and replacing wheat flour by lyophilized almond bagasse in the 10% (LYO-10%), in the 15% (LYO-15%) or in the 25% (LYO-25%). dm, dry matter; X_w, water content; WHC, water holding capacity; WAC, water absorption capacity; OAC, oil absorption capacity; ES, emulsifying stability; EA, emulsifying activity. A, treatment; B, replacement percentage. Mean ± standard deviation of three repetitions. Different superscripts letters in the same line indicate statistically significant differences with a confidence level of 95%.

	HAD60-10%	HAD60-15%	HAD60-25%	LYO-10%	LYO-15%	LYO-25%	p-value		
							A	B	A-B
X_w (g/g_{dm})	0.103 ± 0.004^{c}	0.097 ± 0.002^{b}	0.087 ± 0.004^{a}	0.104 ± 0.002^{c}	0.101 ± 0.002^{bc}	0.089 ± 0.002^{a}	0.09	0.00	0.42
Fat (g/g_{dm})	0.091 ± 0.006^{a}	0.159 ± 0.009^{c}	0.19 ± 0.085^{d}	0.094 ± 0.009^{b}	0.161 ± 0.004^{c}	0.189 ± 0.005^{d}	0.00	0.00	0.82
Ashes (g/g_{dm})	0.0206 ± 0.0004^{a}	0.0205 ± 0.0002^{a}	0.0202 ± 0.0002^{a}	0.0203 ± 0.0001^{a}	0.0204 ± 0.0002^{a}	0.0202 ± 0.0003^{a}	0.45	0.20	0.98
Bulk density (g_{dm}/mL)	0.787 ± 0.009^{d}	0.784 ± 0.002^{cd}	0.771 ± 0.006^{ab}	0.774 ± 0.005^{bc}	0.770 ± 0.005^{ab}	0.762 ± 0.007^{a}	0.03	0.10	0.57
WHC (g_w/g_{dm})	0.62 ± 0.07^{a}	0.61 ± 0.02^{a}	0.58 ± 0.05^{a}	0.86 ± 0.02^{b}	0.91 ± 0.01^{b}	0.85 ± 0.04^{b}	0.00	0.44	0.30
WAC (g_w/g_{dm})	0.708 ± 0.015^{a}	0.71 ± 0.02^{a}	0.72 ± 0.02^{b}	0.72 ± 0.05^{b}	0.73 ± 0.02^{c}	0.74 ± 0.009^{d}	0.15	0.02	0.28
OAC (g_o/g_s)	0.20 ± 0.02^{ab}	0.18 ± 0.02^{a}	0.182 ± 0.007^{a}	0.22 ± 0.03^{b}	0.182 ± 0.005^{a}	0.193 ± 0.006^{ab}	0.00	0.00	0.00
ES (%)	0.7 ± 0.1^{a}	0.7 ± 0.1^{a}	1.8 ± 0.1^{c}	1.1 ± 0.1^{b}	1.8 ± 0.1^{c}	3.6 ± 0.1^{d}	0.00	0.00	0.00
EA (%)	1.8 ± 0.1^{a}	1.8 ± 0.1^{a}	3.6 ± 0.1^{b}	3.6 ± 0.1^{b}	3.6 ± 0.1^{b}	7.1 ± 0.1^{c}	0.00	0.00	0.00

Regarding water absorption capacity (WHC) and water absorption capacity (WAC), significant differences were observed between drying methods, with higher values in mixtures containing freeze-dried powder. As previously mentioned, WHC and WAC are influenced by particle size, suggesting that mixtures containing freeze-dried almond powders will exhibit increased interaction with water (WHC and WAC), as water molecules can penetrate to the larger spaces (Bai et al., 2021). However, it is important to note that, despite the percentage of substitution with almond bagasse powder, the water interaction properties did not exhibit significant improvements. This could be attributed to the fact that the substitution percentages with almond powders are not sufficiently high. Furthermore, the high fat content present in almonds could impede the interaction with water molecules (Martins et al., 2017).

Significant differences ($p \leq 0.05$) were evident in oil interaction properties, including oil absorption capacity (OAC), emulsifying activity (EA) and stability (ES), concerning both the percentage of substitution and the drying method. These properties tended to improve as the substitution percentage increased. It could be attributed to the high protein content present in almond bagasse powder, likely accounting for its remarkable characteristics in oil interaction. Comparable outcomes were noted with dehydrated almond bagasse powder (Duarte et al., 2023). Regarding the dehydration method, blends containing freeze-dried powder displayed higher values compared to those dried using hot air. This effect is attributable to the freeze-drying process, which intensifies structural damage. Consequently, it could lead to the fracture of complex molecules, increasing the availability of hydrophilic and hydrophobic groups for enhanced interaction, thereby improving the oil interaction properties (Özdemir et al., 2022). The use of blends as opposed to wheat flour in the industry could offer several advantages. Firstly, these blends will enhance the

nutritional profile by incorporating polyunsaturated fatty acids, vitamins, and minerals (Laelago et al., 2023; Schmiele et al., 2017). Moreover, they will provide an opportunity to enhance dough functionality, thereby improving factors such as water and oil interaction properties (Harasym et al., 2020; Villanueva et al., 2018). Additionally, their utilization could potentially decrease production costs, contingent upon the specific product used (Martins et al., 2017). Lastly, they will enable the creation of a broader range of products with distinct properties, thereby augmenting versatility in the production line (Martins et al., 2017). However, the utilization of these blends will also come with certain drawbacks. For instance, the complexity involved in formulating, issues related to labelling and consumer perception or the potential for these mixtures to generate undesirable odours and flavours in the final product, which are a crucial consideration.

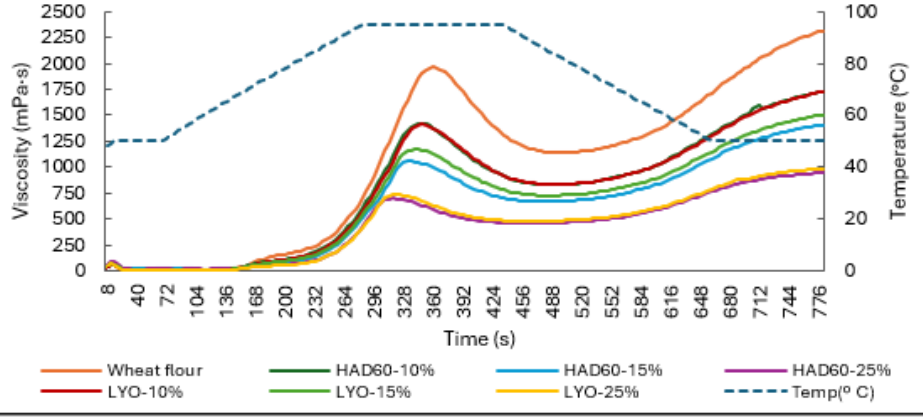
3.3. Pasting and rheological properties of the hydrated blends

Figure 3.5 shows pasting temperature, pasting curves and the pasting properties of the blends prepared replacing wheat flour by hot air dried or lyophilized almond bagasse. The measured parameters included peak viscosity (PV), trough viscosity (TV), breakdown viscosity (BV), final viscosity (FV), setback viscosity (SV), peak time, and pasting temperature. It can be observed that replacing wheat flour by almond bagasse powder had, in all cases, a noteworthy effect on viscosities measured along pasting process. Although reducing the viscosity may improve the ease of handling in the manufacturing process, it will alter the textural and sensory properties of the final product.

The most significant differences are a decrease in BV and pasting temperature. Also, a slight advance in the time at which maximum PV is observed. The PV is an indication of the thickening power of the sample, the higher the peak viscosity, the

higher the thickening power (Chinma et al., 2013). The low peak viscosity values of the blends may be suitable for products requiring low gel strength and elasticity. The differences can be attributed to the reduction in starch content and the increase in fibre and fat content as the replacing percentage of wheat flour by almond bagasse powder increase. Starch granules together with gluten proteins are the main components of wheat flour with the ability to retain water and form a firm structure with the ability to swell, resulting in high dough viscosity. Replacing part of the wheat flour with almond powder, which is rich in insoluble fibre and fat, considerably weakens the gel structure and at the same time hinders the ability to interact with fat.

Although pasting temperature has been reported to be related to water binding capacity, it does not seem to be related for these blends, as WHC is higher in blends with lyophilized almond bagasse than in wheat flour. It may be that the high fat content determines the relationship between temperature and WHC. However, there was no significant change in the structural characteristics of the gel formed by mixing the wheat flour with the almond bagasse powder as, in all cases, the shape of the curve is maintained. It is, the structure from the starch molecules and proteins in the wheat flour continues to determine the behaviour during pasting, although this results in a softer dough as the percentage of wheat flour replaced by almond bagasse powder increases.



	Wheat flour	HAD60-10%	HAD60-15%	HAD60-25%	LYO-10%	LYO-15%	LYO-25%	p-values		
								A	B	A-B
PV (mPa·s)	1964 ± 7^{c}	1409.5 ± 0.7^{d}	1054 ± 4^{b}	679 ± 26^{a}	1413 ± 95^{d}	1182 ± 69^{c}	734 ± 33^{a}	0.00	0.06	0.28
TV (mPa·s)	1142 ± 3^{e}	816 ± 5^{b}	579 ± 122^{a}	457 ± 47^{a}	833 ± 47^{b}	725 ± 43^{b}	478 ± 21^{a}	0.15	0.00	0.27
BV (mPa·s)	822 ± 4^{e}	592 ± 3^{d}	375 ± 23^{b}	219.5 ± 0.7^{a}	580 ± 49^{d}	458 ± 26^{c}	256 ± 13^{a}	0.04	0.00	0.09
FV (mPa·s)	2302 ± 9^{d}	1722 ± 27^{c}	1409 ± 8^{b}	873 ± 3^{a}	1719 ± 113^{c}	1498 ± 52^{b}	983 ± 43^{a}	0.08	0.00	0.45
SV (mPa·s)	1160 ± 6^{d}	891 ± 10^{c}	724 ± 20^{b}	458 ± 13^{a}	886 ± 66^{c}	774 ± 9^{b}	505 ± 23^{a}	0.12	0.00	0.42
Peak time (min)	6.0 ± 0.1^{c}	5.8 ± 0.1^{bc}	5.4 ± 0.2^{a}	5.3 ± 0.1^{a}	5.8 ± 0.1^{bc}	5.7 ± 0.2^{b}	5.3 ± 0.1^{a}	0.14	0.00	0.3
Pasting Temp (°C)	84.73 ± 0.04^{a}	86.0 ± 0.6^{ab}	86.45 ± 0.07^{bc}	88.4 ± 7^{c}	86.0 ± 0.6^{ab}	86.0 ± 0.5^{ab}	87.7± 0.5^{c}	0.00	0.00	0.00

Figure 3.5. Pasting temperature, pasting curves and pasting properties of the blends prepared replacing wheat flour by hot air dried almond bagasse in the 10% (HAD60-10%), in the 15% (HAD60-15%) or in the 25% (HAD60-25%); and replacing wheat flour by lyophilized almond bagasse in the 10% (LYO-10%), in the 15% (LYO-15%) or in the 25% (LYO-25%). PV, peak viscosity; TV, trough viscosity; BV, breakdown viscosity; FV, final viscosity; SV, setback viscosity. A, treatment; B, replacement percentage. Mean ± standard deviation of three repetitions. Different superscripts letters in the same line indicate statistically significant differences with a confidence level of 95%.

In relation to the effect of the dehydration treatment applied to the almond bagasse, it can be considered negligible since the corresponding curves appear practically superimposed, regardless of the percentage of substitution applied. These results are consistent with the results obtained for the water interaction properties of the blends, which show only slightly different values compared to those of wheat flour.

The viscoelastic properties of the different blends are depicted in Figure 3.6. An oscillatory dynamic test was applied to analyse this behaviour. Both the elastic modulus (G') and the viscous modulus (G'') exhibited a slight increase as the frequency increased, indicating a dependency on this variable. Across all the tested blends, G' was consistently higher than G'' (G' > G''), suggesting a prevalence of the elastic component over the viscous one. The control (Figure 3.6A and 3.6B) exhibited the highest values of the elastic modulus G', while the lowest values were observed in the HAD60-25% and LYO-25% mixtures. Similarly, the trend seen in the elastic modulus was mirrored in the viscous modulus G'', with lower values also found in the HAD60-25% and LYO-25% blends. There is a clear impact of substitution evident in both moduli, as the substitution percentage increases, both G' and G'' tend to decrease. This decline can be attributed to the interaction between water and the proteins present in wheat flour (gluten). Gluten plays a crucial role in imparting elasticity and viscosity to the dough (Van Der Borght et al., 2005). Therefore, if the blends contain a reduced amount of wheat flour, their elasticity (G') and viscosity (G'') will be lower.

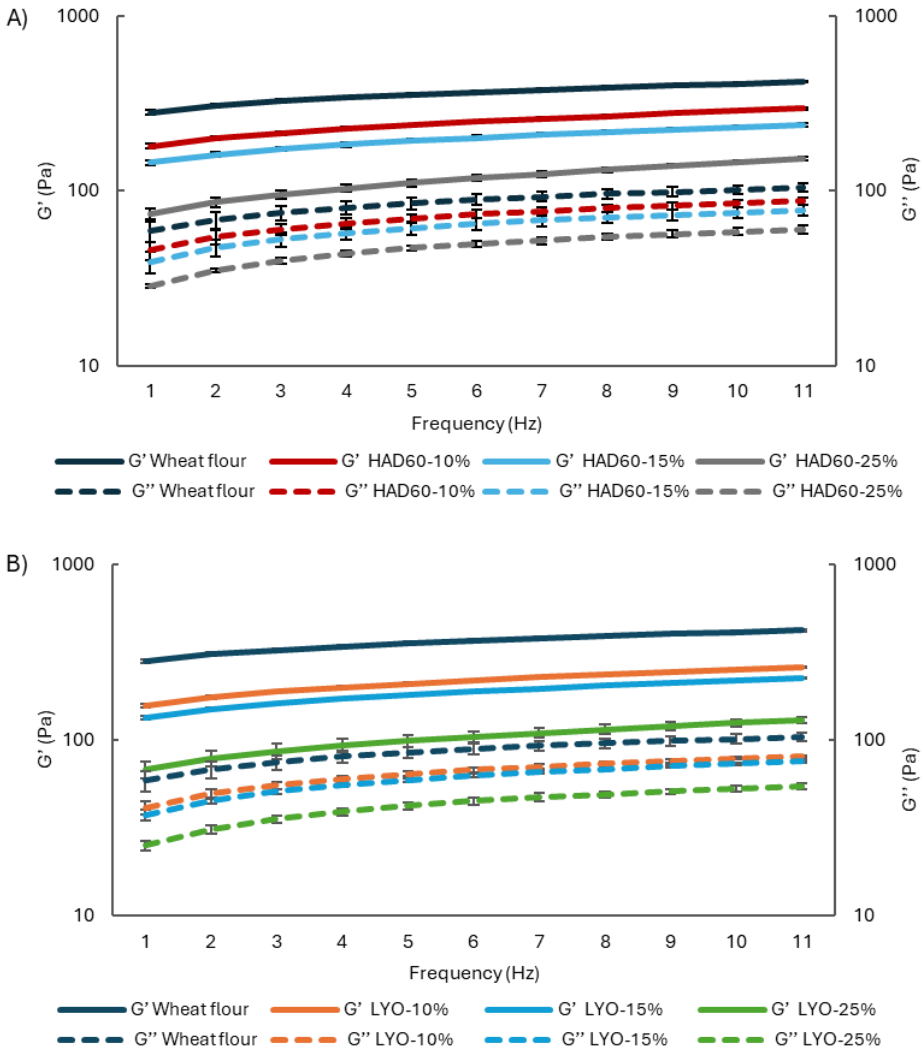


Figure 3.6. Elastic modulus (G') and viscous modulus (G'') of the blends prepared replacing wheat flour by hot air dried almond bagasse (A) in the 10% (HAD60-10%), in the 15% (HAD60-15%) or in the 25% (HAD60-25%); and replacing wheat flour by lyophilized almond bagasse (B) in the 10% (LYO-10%), in the 15% (LYO-15%) or in the 25% (LYO-25%). Mean ± standard deviation of three repetitions.

The textural properties of the hydrated blends with varying percentages of almond bagasse powder substitution, are presented in Figure 3.7. According to the texture measurements, it was observed that all the gels resisted the double compression and could restore their original height and shape. However, the results indicated that the force needed to deform the gels containing almond bagasse powder blends (HAD60 and LYO) was lower compared to the control (wheat flour gel).

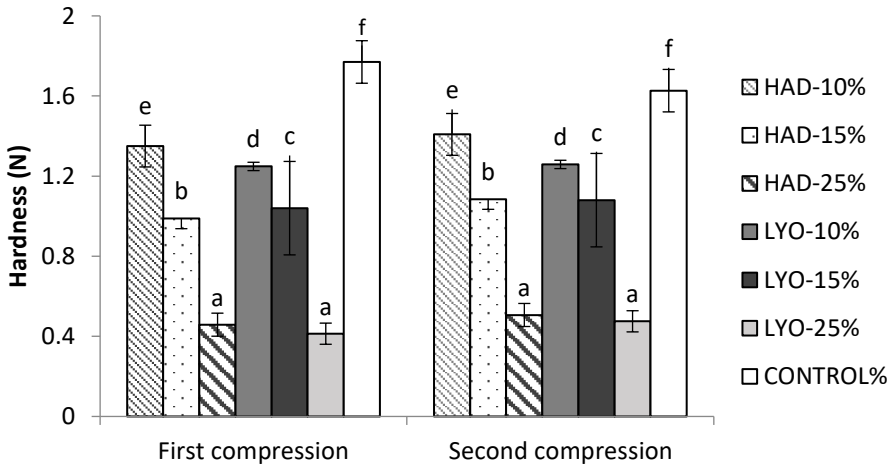


Figure 3.7. Textural properties of the hydrated blends prepared replacing wheat flour (CONTROL) by hot air dried almond bagasse in the 10% (HAD60-10%), in the 15% (HAD60-15%) or in the 25% (HAD60-25%); and replacing wheat flour by lyophilized almond bagasse in the 10% (LYO-10%), in the 15% (LYO-15%) or in the 25% (LYO-25%). Mean ± standard deviation of three repetitions. Different indicate statistically significant differences with a confidence level of 95%.

An increase in the substitution percentage correlated with a decrease in gel hardness. This decrease might be linked to the retrogradation of starch, enabling the formation of a more gelatinous and soluble structure when mixed with water (Dhen et al., 2017). Furthermore, this reduction in hardness could stem from the

reorganization of amylose present in wheat flour, as it plays a crucial role in gel structure formation. This, coupled with the fact that stiffness correlates with amylose content, could elucidate the observed variation (Morris et al., 1990). The decline in stiffness might be due to the reduced quantity of wheat flour owing to the substitution of almond bagasse powder. Previous studies have reported analogous outcomes when substituting apricot kernels for wheat flour, demonstrating a decrease in hardness and the energy required to deform the gel (Dhen et al., 2017).

3.4. Physical properties and antiradical capacity of biscuits prepared with wheat flour and with the different blends of wheat flour and almond bagasse powder.

Table 3.10 shows the absolute and relative weights, dimensions and specific volumes of the biscuits prepared with wheat flour and with the different blends of wheat flour and almond bagasse powder. Both the treatment applied, and the percentage of substitution had a significant effect on the weight and dimensions of the biscuits and, consequently, on the specific volume. However, the differences were not very large and although in all cases they implied a decrease in weight and volume (both thickness and diameter decrease), in some cases the specific volume increased; the biscuits obtained with the blend containing freeze-dried almond powder or hot air-dried almond powder at 25% had a higher specific volume and were therefore less compact. Although pasting and rheological analysis showed a lower viscosity and hardness for the hydrated mixtures containing almond bagasse powder, this did not have a major impact on their suitability for biscuit preparation. The incorporation of almond bagasse powder significantly weakened the structure of the gel formed, but its firmness was sufficient to provide a structured biscuit with a similar specific volume to that obtained with wheat flour.

The colour evaluation of the different formulated biscuits was also carried out, and the CIE-L*a*b* values together with the colour differences (ΔE) compared to the control biscuits, are presented in Table 3.10. A significant decrease in the L* parameter can be observed in all biscuit formulations that have a percentage of almond bagasse powder substitution, resulting in slightly darker biscuits compared to the control. In addition, a significant increase was observed in the a* coordinate, which was slightly higher as the percentage of substitution with almond bagasse powder increased, while no significant differences were observed in the b* coordinate. Consequently, these changes were reflected in colour differences (ΔE) following the same increasing trend as the percentage of substitution with almond bagasse powder increased, with values between 3 and 5 that could be perceptible to the human eye (Bodart et al., 2008; Made et al., 2020). Finally, considering the different drying methods, no significant differences were observed between hot air drying at 60 °C and lyophilisation for each percentage of flour substitution.

Table 3.10. Absolute and relative weights, dimensions, specific volumes and CIE-L*a*b* coordinates of the biscuits prepared with wheat flour and with the blends prepared by replacing wheat flour by hot air dried almond bagasse in the 10% (HAD60-10%), in the 15% (HAD60-15%) or in the 25% (HAD60-25%); and replacing wheat flour by lyophilized almond bagasse in the 10% (LYO-10%), in the 15% (LYO-15%) or in the 25% (LYO-25%); and colour differences (ΔE) compared to the wheat flour biscuits. EV, specific volume; RW, relative weight; RD, relative diameter; RT, relative thickness; REV, relative specific volume. A, treatment; B, replacement percentage. Mean ± standard deviation of three repetitions. Different superscripts letters in the same line indicate statistically significant differences with a confidence level of 95%.

	Wheat flour	HAD60-10%	HAD60-15%	HAD60-25%	LYO-10%	LYO-15%	LYO-25%	p-value		
								A	B	A-B
Weight (g)	8.5 ± 0.4^{d}	8.01 ± 0.14^{cd}	7.4 ± 0.7^{bc}	7.43 ± 0.15^{bc}	6.7 ± 0.2^{ab}	6.3 ± 0.5^{a}	7.2 ± 1.4^{bc}	0.00	0.08	0.07
Diameter (mm)	52 ± 2^{d}	48 ± 3^{abc}	49.2 ± 0.7^{bcd}	51 ± 3^{cd}	46 ± 2^{ab}	46 ± 2^{a}	49.7 ± 0.9^{bcd}	0.06	0.04	0.50
Thickness (mm)	5.43 ± 0.13^{b}	5.5 ± 0.5^{b}	4.81 ± 0.11^{ab}	4.9 ± 0.2^{ab}	4.68 ± 0.08^{a}	4.9 ± 0.5^{ab}	5.1 ± 0.7^{ab}	0.47	0.69	0.11
EV (cm^3/g)	1.36 ± 0.37^{d}	1.24 ± 0.25^{cd}	1.24 ± 0.35^{abc}	1.36 ± 0.12^{bc}	1.16 ± 0.25^{a}	1.29 ± 0.06^{ab}	1.37 ± 0.43^{bcd}	0.09	0.22	0.16
RW	1.00 ± 0.01^{d}	0.94 ± 0.06^{cd}	0.86 ± 0.04^{bc}	0.87 ± 0.04^{bc}	0.79 ± 0.06^{ab}	0.73 ± 0.06^{a}	0.88 ± 0.12^{bc}	0.01	0.16	0.14
RD	1.00 ± 0.01^{d}	0.92 ± 0.05^{abc}	0.95 ± 0.05^{bcd}	0.98 ± 0.06^{cd}	0.89 ± 0.01^{ab}	0.88 ± 0.03^{a}	0.96 ± 0.04^{bcd}	0.08	0.04	0.51
RT	1.00 ± 0.01^{bc}	1.0 ± 0.2^{c}	0.89 ± 0.04^{ab}	0.91 ± 0.02^{abc}	0.86 ± 0.02^{a}	0.91 ± 0.08^{abc}	0.95 ± 0.11^{abc}	0.43	0.66	0.09
REV	1 ± 0.01	0.91 ± 0.11^{a}	0.91 ± 0.05^{a}	1.02 ± 0.08^{a}	0.85 ± 0.09^{a}	0.95 ± 0.09^{a}	1.007 ± 0.011^{a}	0.26	0.52	0.38
				Colour						
L	62.4 ± 0.4^{e}	58.9 ± 0.2^{bc}	58.4 ± 0.2^{ab}	57.8 ± 0.3^{a}	59.8 ± 0.6^{d}	59.2 ± 0.7^{cd}	58.0 ± 0.3^{a}	0.01	0.00	0.30
a*	12.5 ± 0.2^{a}	13.6 ± 0.4^{b}	13.90 ± 0.08^{bc}	15.0 ± 0.4^{d}	13.4 ± 1.0^{b}	13.65 ± 0.09^{b}	14.6 ± 0.2^{cd}	0.29	0.00	0.90
b*	33.5 ± 0.3^{a}	33.7 ± 0.3^{a}	33.82 ± 0.11^{a}	34 ± 2^{a}	34.1 ± 0.7^{a}	33.1 ± 0.5^{a}	34.4 ± 0.3^{a}	0.23	0.11	0.89
C	35.8 ± 0.4^{a}	35.9 ± 0.2^{ab}	35.85 ± 0.09^{ab}	36 ± 1^{ab}	36.7 ± 1.0^{bc}	35.8 ± 0.4^{ab}	37.4 ± 0.2^{c}	0.01	0.01	0.30
ΔE	-	3.7 ± 0.3^{ab}	4.3 ± 0.2^{bc}	5.5 ± 0.3^{d}	2.9 ± 0.9^{a}	3.5 ± 0.7^{ab}	5.0 ± 0.3^{cd}	0.01	0.00	0.79

Antioxidant activity assessments using the DPPH and ABTS methods of biscuits were shown at Figure 3.8. Significant differences were noted in both the drying method (hot air at 60°C and freeze-drying) and the percentage of almond bagasse powder substitution in the biscuit production. Moreover, a discernible trend was observed. As the percentage of almond bagasse powder substitution increased (10, 15, and 25%), the DPPH antiradical activity rose within the range of 1.01 to 1.12 mg Trolox/g dry sample in the samples dried using hot air at 60°C. Conversely, in the freeze-dried samples, an increase ranging from 0.87 to 0.97 mg Trolox/g dry sample was recorded, with higher antiradical DPPH activity in the samples dried using hot air. This discrepancy might be attributed to reactions due to the drying temperature. High temperatures could potentially induce the generation of Maillard reaction by-products, known for their remarkable antioxidant capacity (Nooshkam et al., 2019). Conversely, while assessing antiradical activity using the ABTS method, no significant differences were noted concerning the drying method or the percentage of almond bagasse powder substitution. Nevertheless, it's noteworthy that, despite the absence of significant differences, an upward trend was observed as the percentage of almond bagasse powder substitution increased across various formulations.

Additionally, we evaluated the total phenolic content in different biscuit formulations. As depicted in Figure 3.8, coinciding with the ABTS antiradical activity determination, an increase in total phenolic content was evident with an increased percentage of almond bagasse powder in the biscuit formulation. No significant differences were observed between the drying methods. Importantly, in both the assessment of antioxidant activity and total phenolic content, control samples made solely with 100% wheat flour exhibited the lowest values compared to samples containing almond bagasse powder substitution. This difference is attributed to the

likelihood that wheat flour may possess a less diverse profile of antioxidant compounds (Ragaee et al., 2011).

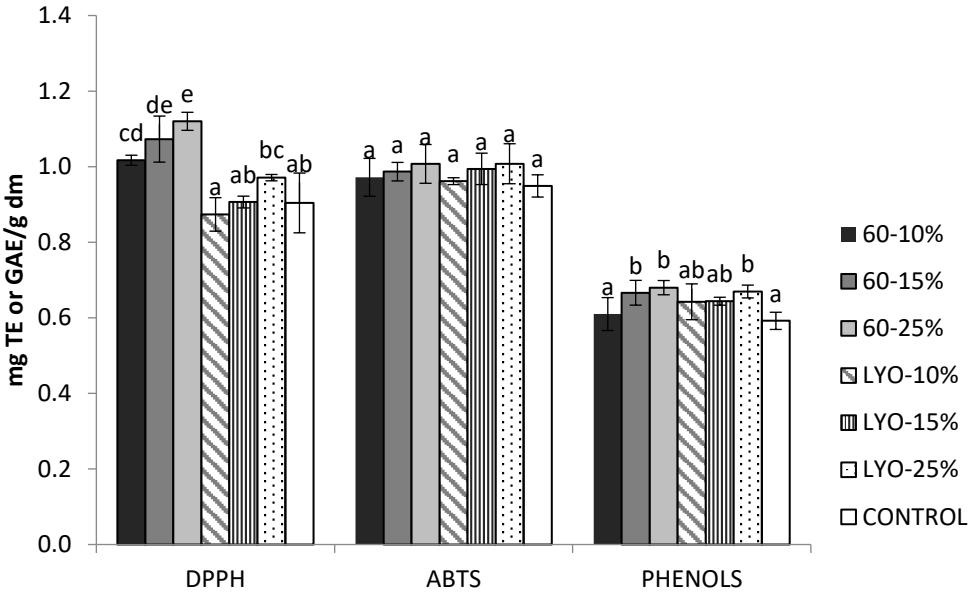


Figure 3.8. Total phenols content and antioxidant activity of biscuits prepared replacing wheat flour (CONTROL) by hot air dried almond bagasse in the 10% (HAD60-10%), in the 15% (HAD60-15%) or in the 25% (HAD60-25%); and replacing wheat flour by lyophilized almond bagasse in the 10% (LYO-10%), in the 15% (LYO-15%) or in the 25% (LYO-25%). Mean ± standard deviation of three repetitions. Different letters indicate statistically significant differences with a confidence level of 95%.

4. Conclusions

The absence of starch and the high fat content conditioned suitability of almond bagasse powder as a substitute for wheat flour in the formulation of bakery products. The water absorption and retention capacity were significantly different in the blends containing powders obtained by different dehydration methods, with higher values obtained in the blends containing the freeze-dried powder.

Although rheological analyses showed lower viscosity and hardness for the hydrated mixtures containing almond bagasse powder, this did not have a major impact on their suitability for biscuit production. The incorporation of almond bagasse powder significantly weakened the structure of the gel formed, but its hardness was sufficient to provide a structured biscuit with a specific volume like that obtained with wheat flour and with a higher anti-radical capacity.

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CONCLUSIONES CAPÍTULO I

Tanto el secado por aire caliente a 60 y 70 °C como la liofilización, combinadas con un triturado moderado, resultaron ser métodos adecuados para producir un polvo vegetal a partir del bagazo resultante del proceso de obtención de la bebida vegetal de almendra.

La cinética del proceso de secado por aire caliente mostró el secado en dos periodos de velocidad, obteniéndose buenos coeficientes de correlación cuando los datos se ajustaron al modelo difusional de Fick o a un modelo empírico lineal. La cinética más rápida a 70 °C conllevaría una mayor eficiencia industrial.

En términos de composición se obtuvo un polvo proteico con elevado contenido en grasa y fibra insoluble. En cuanto a la estructura macromolecular, en todos los casos resultó un polvo con partículas grandes y ligeramente apelmazadas, si bien la liofilización permitió obtener una distribución más uniforme del tamaño de partícula. Ambas características, composición y distribución del tamaño de partícula, condicionaron en gran medida las propiedades de interacción con el agua y con el aceite.

El método de secado no afectó las propiedades de solubilidad e higroscopicidad de los polvos que resultaron ser baja y moderada respectivamente. Sin embargo, la capacidad de retención de agua y las propiedades de interacción con el aceite fueron mejores en los polvos liofilizadas. Estos efectos condicionarán la estabilidad física durante el almacenamiento y las aplicaciones industriales más recomendables en cada caso.

En los tratamientos de secado con aire caliente, los daños estructurales y la presencia de oxígeno a 70 °C provocaron una mayor degradación de los compuestos fenólicos, si bien la capacidad antirradical no se vio afectada por el método de secado empleado.

Tanto el secado por aire caliente como la liofilización aseguraron la estabilidad física y microbiológica del polvo durante tres meses, incluso en condiciones de almacenamiento acelerado. Además, el incremento moderado en el índice de peróxidos y la acidez a temperatura ambiente es previsible de no producir alteraciones significativas en las propiedades del producto final.

El almacenamiento aumentó el contenido de polifenoles y la actividad antirradical debido a cambios estructurales provocados por la deshidratación, así como a la formación de compuestos asociados a las reacciones de Maillard durante el secado por aire caliente. Para poder explicar de forma precisa estos efectos sería necesario llevar a cabo análisis bioquímicos más específicos relacionados con la liberación de compuestos polifenólicos específicos, la cinética de reacciones degradativas y las interacciones entre compuestos en cada tratamiento.

La ausencia de almidón y el elevado contenido en grasa condicionaron la idoneidad del polvo de bagazo de almendra como sustituto de la harina de trigo en la formulación de productos de panadería. La capacidad de absorción y de retención de agua resultaron significativamente diferentes en las mezclas que incluían polvos obtenidos con métodos de deshidratación diferentes, obteniéndose valores más altos en las mezclas que contenían el polvo liofilizado.

Aunque los análisis reológicos mostraron una viscosidad y una dureza menores para las mezclas hidratadas que contenían polvo de bagazo de almendra, esto no

tuvo un impacto importante en su idoneidad para la producción de galletas. La incorporación de bagazo de almendra en polvo debilitó significativamente la estructura del gel formado, pero su firmeza fue suficiente para proporcionar una galleta estructurada con un volumen específico similar al obtenido con harina de trigo y con una mayor capacidad antirradical.

CAPÍTULO II

CAPÍTULO II: DIGESTIÓN GASTROINTESTINAL Y FERMENTACIÓN COLÓNICA *IN VITRO* DEL BAGAZO DE ALMENDRA. INFLUENCIA DE LA DESHIDRATACIÓN EN EL PERFIL POLIFENÓLICO, ESTRUCTURA DE LA MICROBIOTA Y PRODUCCIÓN DE ÁCIDOS GRASOS DE CADENA CORTA.

ARTÍCULO 4

Duarte, S., Puchades, A., Jiménez-Hernández, N., Betoret, E., Gosalbes, M. J., & Betoret, N. (2023). Almond (Prunus dulcis) Bagasse as a Source of Bioactive Compounds with Antioxidant Properties: An In Vitro Assessment. Antioxidants, 12(6), 1229. **DOI:** https://doi.org/10.3390/antiox12061229

ARTÍCULO 5

Duarte, S., N., Betoret, E., & Betoret, N. (2024). Effect of Dehydration on the Production of Short and Branched Chain Fatty Acids (SCFA and BCFA) in Almond Bagasse during Colonic Fermentation *in vitro*. **En preparación**.

RESUMEN CAPÍTULO II

La almendra (*Prunus dulcis*) constituye una excelente fuente de fibra dietética, ácidos grasos monoinsaturados, proteína, antioxidantes y compuestos fenólicos. Los fenoles son compuestos químicos que poseen propiedades antioxidantes y beneficios para la salud. Algunos de los compuestos fenólicos específicos en la almendra son el ácido gálico, la quercetina, el ácido cafeico, la epicatequina, la catequina, entre otros, pudiendo variar la concentración de dichos compuestos dependiendo de la variedad de almendra. Durante la fabricación de la bebida vegetal de almendra, algunos componentes del grano se solubilizan y se trasfieren al agua. Sin embargo, una cantidad considerable de los compuestos queda retenida en el residuo sólido o bagazo de almendra.

Como se ha mencionado a lo largo del documento, el residuo procedente de la obtención de la bebida vegetal de almendra (bagazo de almendra), aunque es considerado un material de bajo valor, contiene gran cantidad de compuestos bioactivos con propiedades antioxidantes, lo que lo convierte en un producto óptimo para ser revalorizado. Sin embargo, para garantizar que estos compuestos ejerzan un efecto beneficioso en la salud, es necesario conocer su bioaccesibilidad tras la digestión gastrointestinal y posteriormente, determinar su efecto sobre la microbiota tras la fermentación colónica. Por consiguiente, este segundo capítulo comprende los estudios relacionados con la influencia del proceso de deshidratación en la digestión gastrointestinal *in vitro* (fase oral, gástrica e intestinal) y fermentación colónica, con especial atención sobre los compuestos bioactivos específicos, microbiota fermentativa del colon y los ácidos grasos de cadena corta.

El primer trabajo de investigación de este capítulo evaluó el efecto de las condiciones de deshidratación y la digestión gastrointestinal *in vitro* sobre la

capacidad antirradical, fenoles totales y contenido en polifenoles específicos en el bagazo de almendra. Además, se evaluó el efecto sobre la comunidad microbiana fermentativa y el contenido de polifenoles tras la fermentación colónica. El bagazo de almendra se obtuvo a partir del remojo y triturado de las almendras enteras, seguido de la deshidratación (secado por aire caliente a 60 o 70 °C y liofilización) y una molienda para obtener un polvo estable. La determinación de la capacidad antirradical, fenoles totales y el contenido de diez polifenoles específicos, se llevó a cabo en cada etapa de la digestión *in vitro* (fase oral, gástrica e intestinal) y al final de la fermentación colónica. Además, se determinaron los cambios estructurales en la comunidad bacteriana del colon tras la fermentación colónica, utilizando inóculos de adultos sanos, de los diferentes bagazos triturados.

Según los resultados obtenidos, el proceso de deshidratación influye significativamente en la capacidad antioxidante. El proceso de liofilización dio lugar a polvos con mayor contenido en fenoles totales, así como un mayor contenido de polifenoles específicos. Por el contrario, las muestras secadas por aire caliente tuvieron una mayor degradación de los compuestos bioactivos antes mencionados. Esto podría deberse a que las altas temperaturas provocan daño estructural, y además, la presencia de oxígeno favorece la degradación de los compuestos antioxidantes. Por otro lado, durante la digestión gastrointestinal *in vitro* se redujo la cantidad de compuestos polifenólicos específicos debido, posiblemente, a la interacción entre compuestos bioactivos y macromoléculas. En cuanto a los fenoles totales y la capacidad antioxidante, aumentaron progresivamente durante las etapas gástrica, intestinal y colónica, sin diferencias significativas entre los métodos de deshidratación. Por último, en el análisis de la microbiota fermentativa, los sustratos afectaron la estructura de la comunidad microbiana, siendo significativamente diferente en las muestras control. La deshidratación del bagazo de almendra tiene un

claro efecto sobre la microbiota colónica, favoreciendo el crecimiento de varios géneros productores de ácidos grasos de cadena corta (SCFA) como es el caso del Butyrivibrio. Los SCFA tienen varios efectos beneficiosos en el organismo, sirviendo como fuente de energía para las células del colon o favoreciendo las propiedades antiinflamatorias. Los SCFA son productos de la fermentación de carbohidratos no digeribles, aunque también se pueden presentar en menor medida los ácidos grasos de cadena ramificada (BCFA) como producto de la fermentación de proteínas.

Teniendo en cuenta que la fermentación colónica favoreció el crecimiento de bacterias productoras de SCFA, se planteó en el segundo trabajo de investigación evaluar el impacto del proceso de deshidratación de las muestras sobre la producción de SCFA y BCFA tras la etapa de fermentación colónica *in vitro*. Los SCFA determinados después de la fermentación colónica del bagazo de almendra deshidratado (aire caliente y liofilización) incluyeron el ácido acético, el ácido propiónico, el ácido butírico y el ácido valérico, mientras que los BCFA determinados fueron el ácido isovalérico y el ácido isobutírico.

Los resultados obtenidos indicaron que, tanto en la fermentación colónica de las muestras deshidratadas por aire caliente como en las liofilizadas, predominó la producción de BCFA frente a la de SCFA. Probablemente estos resultados se debieron al alto contenido de proteína en el bagazo de almendra, y a la acción de bacterias como las eubacterias (Megasphaera elsdenii, bacteroides sacarolíticos, bacteroides asacarolíticos) y una variedad de cocos anaerobios Grampositivos que promueven la producción de BCFA. Por otro lado, aunque en menor medida, el bagazo también estimuló la producción de butirato debido a la presencia de Butyrivibrio, lo que podría tener potenciales beneficios para la salud humana. Finalmente, la producción

de ácido valérico también se observó como resultado de la fermentación de aminoácidos.

antioxidants

MDPI

Article

Almond (*Prunus dulcis*) Bagasse as a Source of Bioactive Compounds with Antioxidant Properties: An In Vitro Assessment

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ARTÍCULO 4: Almond (*Prunus dulcis*) bagasse as a source of bioactive compounds with antioxidant properties: An *in vitro* assessment.

ABSTRACT

The presence of components of nutritional interest makes fresh almond bagasse an interesting by-product for obtaining functional ingredients. Stabilization through a dehydration process is an interesting option for its integral use ensuring its conservation and management. Subsequently, it can be turned into powder facilitating its use as an ingredient. The aim of this paper was to determine the effect of hot air drying at 60 and 70 °C and lyophilization on the release of phenolic components and antiradical capacity in *in vitro* gastrointestinal digestion and colonic fermentation as well as on growing microbiota composition applying high throughput sequencing. The novelty of this study lies on this holistic approach; considering both technological and physiological aspects related to gastrointestinal digestion and colonic fermentation, will provide the best conditions for functional foods. The results obtained showed that lyophilization provides a powder with a total phenol content and antiradical capacity higher than hot air drying. Furthermore, in dehydrated samples, both *in vitro* digestion and colonic fermentation revealed a phenol content and anti-radical capacity superior to those existing in undigested products. In addition, after colonic fermentation, beneficial bacteria species have been identified. Obtaining powders from almond bagasse is presented as an interesting opportunity for the valorisation of this by-product.

Keywords: bioactive compounds; almond by-product; antiradical capacity; *in vitro* digestion; colonic fermentation, gut microbiota, metagenomics

1. Introduction

Consumer trends and agri-food industry have evolved to more sustainable and nutritional diets [1]. Plant-based products are being extensively assessed as sustainable alternatives to animal protein and as an important source of bioactive compounds [2]. The consumption of cereal, legume, or nut beverages such as oat, soy or almond vegetable drinks has been increased in recent years [3]. Particularly, in Mediterranean countries, the almond (*Prunus dulcis*) vegetal drink has deserved particular attention since it serves as an excellent source of dietary fibres, monounsaturated fatty acids, vitamin E, protein, essential minerals, riboflavin and antioxidants [4]. Extracts of whole almond seed have demonstrated to possess potent free radical scavenging capacities [5]. These activities have been related to the presence of flavonoids and other phenolic compounds [6].

During the process of obtaining almond beverage, many components of the kernel are solubilized and transferred to water in a solid-liquid extraction process. However, a considerable amount is retained in the generated solid residue known as bagasse or cake. Although there are many studies determining the content of macronutrients and bioactive compounds in kernel, hull and shell, no studies have been found providing detailed polyphenolic composition of almond bagasse [6].

On the other hand, the large amount of bagasse generated needs to be managed in order to avoid an important ecological footprint. Finding high value-added alternatives beyond animal feed is a challenge. Due to its high water activity, recovery must first include stabilization of the product. Otherwise, microorganism spoilage and rancidity can become a problem leaving it useless. The combination of grinding and dehydration has been shown to be a suitable processing method for the production of stable powdered ingredients with interesting industrial applications

[7]. This form of processing has been applied to a wide range of fruits and vegetables and there are now many powdered products on the market. In recent years, research in this area has also been extended to several by-products, in particular the effect of processing on the content and bioaccessibility of bioactive compounds has been studied. It is well known that processing conditions can affect structure, functionality and accessibility of bioactive compounds determining their beneficial effect. Furthermore, its particle size and the presence of other macromolecules such as carbohydrates or proteins could influence chemical or physical interactions facilitating or inhibiting the release and absorption of bioactive compounds in the gastrointestinal tract [7].

Furthermore, much research is being done to elucidate the influence of diet on the colonic fermentative microbiota since it plays a fundamental role in the degradation of complex macromolecules and affecting the metabolism of phenolic compounds and secondary metabolites generation with potential beneficial effect on human health [8]. However, in order to ensure the best health effects, the effect of processing, the evolution along the gastrointestinal tract and the influence on the colonic fermentative microbiota must be analysed as a whole. A holistic approach, considering both technological and physiological aspects related to gastrointestinal digestion and colonic fermentation, will provide the best conditions for functional foods. The novelty of this work lies on this holistic approach, including the effect of processing, gastrointestinal digestion and colonic fermentation.

The aim of this paper was to examine the effect of dehydration conditions and *in vitro* gastrointestinal digestion on antiradical capacity, total phenols and specific polyphenol content in almond bagasse. Moreover, the effect on the fermentative

microbial community and the polyphenol content after colonic fermentation have been determined.

2. Materials and Methods

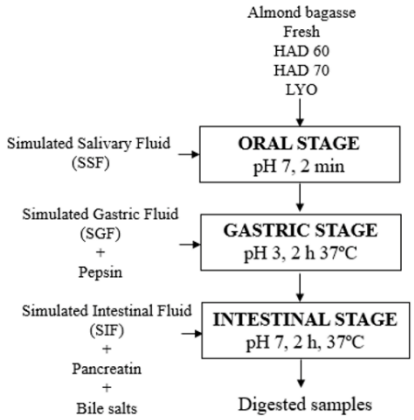
2.1. Production of almond bagasse and powder

Natural almonds were purchased from a local supermarket in Valencia city (Spain) and ground with drinking water at a ratio of 1/9 (w/w). Grinding was performed using a domestic kitchen appliance (Thermomix®, Vorwerk, Spain) at 10,000 rpm for 20 s. The grind was then filtered and therefore the almond bagasse was recovered for further characterization and processing.

To obtain almond bagasse powder, the almond bagasse was homogeneously distributed in plastic grids with a nominal spacing of 2 mm, dehydrated to a water activity lower than 0.3 and finally ground. A convective dryer (Pol-eko Aparatura, Katowice, Poland) with cross-flow air at 60 or 70 °C, for 10 h and 7 h respectively, was used to produce hot air dried bagasse (HAD); and a freeze dryer (Telstar, Lioalta-g) to produce lyophilized one (LYO) from fresh almond bagasse previously frozen at -40 °C for 24 h. Once almond bagasse was dehydrated, the particle size was reduced employing a kitchen appliance (Thermomix®, Vorwerk, Spain). Grinding was carried out at 4,000 rpm for 20 s at 5 s intervals followed by 10,000 rpm for 20 s at 5 s intervals to obtain almond bagasse powder with a coarse granulometry. Finally, powders were stored in opaque glass jars at room temperature for two weeks as maximum.

2.2. Simulation of gastrointestinal digestion *in vitro*.

The methodology proposed by Minekus et al. [9] was followed for the simulation of the oral, gastric, and intestinal digestion stages. According to the protocol, the phases mixed in the successive stages must be kept in a 1:1 ratio (v/v). In this case, 5 g of sample, 5 mL of simulated salivary fluid (SSF), 10 mL of simulated gastric fluid (GSF) and 20 mL of simulated intestinal fluid (SIF) were added. Conditions and procedure are shown in Figure 3.9. Three repetitions for each type of sample (fresh almond bagasse (FRESH), hot air-dried powder at 60 °C (HAD60), hot air dried powder at 70 °C (HAD70) and lyophilized powder (LYO) were performed.



	Salt content (g/L)	SSF(mL/L)	SGF(mL/L)	SIF(mL/L)
KCl	37.3	30.2	27.6	27.2
KH_2PO_4	68	7.4	3.6	3.2
$NaHCO_3$	84	13.6	50	170
NaCl	117	-	47.2	38.4
$MgCl_2(H_2O)_6$	30.5	1	1.6	4.4
$(NH_4)_2CO_3$	48	0.12	2	-
$CaCl_2(H_2O)_2$	44	0.05	0.01	0.08

Figure 3.9. Flowchart, process conditions and composition of the simulated phases for the different stages of the *in vitro* gastrointestinal digestion process. HAD60: hot air dried powder at 60 °C, HAD70: hot air dried powder at 70 °C, and LYO: lyophilized powder.

2.3. *In vitro* colonic fermentation

Faecal samples were collected from healthy adult donors who had not taken antibiotics, prebiotics, or probiotics for three months prior to the assay. The study

was conducted according to the guidelines of the Declaration of Helsinki and the subjects gave their informed consent before they participated in the study. The faecal samples from the donors were pooled together to reduce intra-individual daily variability, and the pool was used to prepare the inoculum (10% w/v). After *in vitro* gastrointestinal digestion of the different samples: fresh bagasse (bagasse), hot air dried powder at 60 °C (HAD60), hot air dried powder at 70 °C (HAD70), and lyophilized powder (LYO), the solid residue (fraction not available for absorption) that is left after removing the supernatant, plus 10% of such digestion supernatant are used as substrate (1% w/v) for fermentation as described by [10]. The colonic fermentation was implemented out in triplicate. Moreover, a control fermentation without a substrate was performed.

2.4. DNA extraction, sequencing and microbial analysis.

Total DNA was extracted from fermentation aliquots in the robotic workstation MagNA Pure LC Instrument (Roche) using the MagNA Pure LC DNA isolation kit III (Bacteria, Fungi) (Roche), following the manufacturer's instructions. The V3-V4 hypervariable region of the 16S rRNA gene was amplified using microbial genomic DNA as template, following the Illumina protocol for 16S Metagenomic Sequencing Library Preparation. Sequencing was performed with the Kit v3 (2 × 230 cycles) in a MiSeq platform (Illumina) at FISABIO-Salud Pública. All the sequences have been deposited in the EBI database under the study number PRJEB61665 with the sample accession numbers: ERSI4956700, ERSI4956701, ERSI4956702, ERSI4956703, ERSI4956704, ERSI4956705, ERSI4956706, ERSI4956707, ERSI4956708, ERSI4956709, ERSI4956710, ERSI4956711, ERSI4956712.

16S rRNA gene reads with low-quality score and short read length as well as potential chimeras were removed using the DADA2 pipeline in the R package [11].

Also, reads were aligned against the human genome (GRCh38.p13) using Bowtie2 (v2.3.5.1) [12] and matches were discarded. The DADA2 pipeline was also used to create the amplicon sequence variants (ASV). The taxonomic information of the 16S rDNA sequences was obtained by similarity comparison using the Basic Local Alignment Search Tool (BLAST) algorithm against the SILVA database (v.138) [13,14]. The analysis of bacterial composition was performed at genus level and PERMANOVA test were performed using the adonis function from vegan library of R package with 600 permutations and the Benjamini-Hochberg procedure for false discovery rate control [15]. Barplots and Canonical Correspondence Analysis (CCA) were generated with in-house R scripts.

Analysis of compositions of microbiomes with bias correction (ANCOMBC) package [16] was applied to identify differentially abundant taxa among substrate fermentations and test significance was determined using the Benjamini-Hochberg procedure for false discovery rate control (q-value).

2.5. Determination of antioxidant compounds and antiradical capacity

For the extraction of antioxidants, 10 mL of a methanol-water mixture was used as a solvent in a ratio of 80:20 (v/v) and mixed with 1 g of sample. After 1 h of magnetic stirring, it was centrifuged (Selecta, "Medrifriger BL-S") at 10,000 rpm for 5 min at 20 °C. Determinations were performed on the supernatant which will be referred to as the extract below.

2.5.1. Total phenol content

The determination of total phenols was carried out following the colorimetric method of Folin-Ciocalteu [17]. In a spectrophotometric cell, 0.125 mL of extract,

0.125 mL of the Folin-Ciocalteu reagent (Sigma-Aldrich) and 0.5 mL of bidistilled water were added in that order and it was allowed to react for 6 min. After this time, 1.25 mL of 7% sodium carbonate (w/v) and 1 mL of distilled water were added. As a reference the extract was replaced by bidistilled water, allowed to react for 90 min. After that, the absorbance was measured at 765 nm in a spectrophotometer (Thermo Scientific, Helios Zeta U / Vis). The results obtained were compared with a standard curve of gallic acid (purity ≥ 98%).

2.5.2. Antiradical capacity by DPPH and ABTS methods

The antioxidant capacity was determined following the DPPH method described by Stratil et al.[18] and Kuskoski et al.[19], with some modifications. Specifically, 0.1 mL of the extract and 2.9 mL of the methanol-DPPH solution were mixed and absorbance was measured at 517 nm in a spectrophotometer (Thermo Scientific, Helios Zeta U/Vis). Trolox was used as the reference standard antioxidant ($C_{14}H_{18}O_4$, purity ≥ 7%, Sigma-Aldrich) and a Trolox calibration curve was obtained for the range of concentrations between 0 and 500 mg/L. The results are expressed as milligrams of Trolox equivalent per gram of dry matter (mg TE/g dm).

The antioxidant activity was also evaluated following the ABTS radical method (2,20-azobis-3-ethyl benzothiazolin-6-sulfonic acid). The methodology proposed by Re et al. [20] was followed. A solution of the acid (7 mM, purity ≥ 99%) was prepared with potassium persulfate (2.45 mM, purity 99.99%) in distilled water, and incubated in darkness at room temperature for 16 h. After that, a dilution with phosphate buffer was made until an absorbance of 0.70 ± 0.02 at 734 nm was reached. Then, in a spectrophotometry cell, 0.1 mL of extract with 2.9 mL of ABTS solution was reacted. As a reference, the sample was replaced by double distilled water. The absorbance

was measured after 0, 3 and 7 min of reaction time at a wavelength of 734 nm in a spectrophotometer (Thermo Scientific, Helios Zeta UV/Vis).

2.5.3. Phenolic compounds by HPLC analysis

Phenolic compounds were extracted following the methodology proposed by Caprioli et al. [21] and Giusti et al. [22]. To perform and acid hydrolysis, 2.5 g of sample were mixed with 7.5 mL of solvent (70:30 ethanol and double distilled water), adjusted the pH to 4 with 2 N hydrochloric acid and transferred to an ultrasonic bath (J.P. Setecta, 3000840) for 2 h at room temperature. Samples were centrifuged at 8,000 g for 15 min, and the extraction was repeated in the solid sample. The supernatants from the two extractions were filtered with a 20 µm PTFE filter and subsequently analysed by HPLC.

For a basic hydrolysis, 14 mL of a solution 2 N sodium hydroxide, 0.01% EDTA 10 mM and 0.1% ascorbic acid was mixed with the pellet obtained after the acid hydrolysis and left overnight to facilitate the release of phenolic ethers or esters. The pH was adjusted to 2 with 6 N hydrochloric acid and centrifuged at 8,000 g for 15 min. The supernatant was mixed with 15 mL of a 50:50 ethyl acetate/diethyl ether solution and centrifuged at 5,400 g for 15 min twice. Supernatants were collected and concentrated on a rotary evaporator (Heidolph) at 26 °C. The resultant was reconstituted with 10 mL methanol and filtered through a 20 µm PTFE filter. Finally, the phenolic fraction was analysed by HPLC.

Samples were processed using a 1200 Series Rapid Resolution HPLC coupled to a Series diode array detector (Agilent, Palo Alto, CA, USA), according to the methodology described by Tanleque-Alberto et al. [23]. Phenolic extracts were separated on a Brisa-LC 5 column Tanleque-Alberto et al. [23]. Mobile phase B was

acetonitrile and mobile phase A was 1% formic acid. The gradients were: 0 min 10% B, 25 min 60% B, 26 min 80% B holding until 30 min, 35 min 10% B holding until 40 min. Working conditions were 30 °C, 0.5 mL/min injection volume and 10 µL flow rate. The different phenolic compounds were identified with the chromatographic retention times of reference standards for each compound at the following wavelengths: Vanillin, 250 nm; 260 nm for 4-hydroxybenzoic acid, Rutin and Quercetin 3-glucoside; 280 nm for epicatechin and chlorogenic acid; 320 nm for p-coumaric acid, Sinapic acid, Ferulic acid and Apeigenin-7-glucoside. Specific compounds were quantified using calibration curves and results were expressed in mg/100 dry sample.

2.6. Statistical analysis

All determinations were done in triplicate. The statistical analysis of the data was performed in a Statgraphics Centurion XVII Software Package, by a simple or multifactorial analysis of variance (ANOVA) at a 95% confidence level ($p \leq 0.05$). Significant differences among groups were determined using the Fisher LSD test.

PERMANOVA test based on a dissimilarity test was applied to evaluate the effect of the external factors on the bacterial composition using the adonis function with 600 permutations from vegan library of R package. The Benjamini-Hochberg procedure was applied for false discovery rate control.

3. Results

3.1. Effect of processing on antiradical capacity and phenol content

In this work, ten of the most common specific polyphenols found in almond kernel were determined in the almond bagasse. The ten polyphenols determined belong to the next three groups: hydroxybenzoic acid derivatives (vanillic acid, 4-hydroxybenzoic acid), hydroxycinnamic acid derivatives (p-coumaric acid, sinapic acid, ferulic acid and chlorogenic acid), flavonoids and derivatives (rutin, quercetin-3-glucoside, quercitrin, epicatechin and apigenin-7-glucoside). Epicatechin was the polyphenol with the highest content (732.7 ± 1.4 mg/ 100 g dry matter) in fresh bagasse followed by vanillic acid (292.3 ± 6.9 mg/ 100 g dry matter), rutin (254.7 ± 1.4 mg/ 100 g dry matter) and sinapic acid (208.4 ± 0.9 mg/ 100 g dry matter).

The effect of dehydration treatment on the polyphenols content is shown in Figure 3.10. Treatment had always a significant effect on the content of polyphenols ($p \leq 0.05$). The effect was different depending on the specific polyphenol considered. In almost all cases, hot air drying resulted in bigger degradation of polyphenols than lyophilization, excepting in rutin (Figure 3.10B) and apigenin-7-glucoside (Figure 3.10B) flavonoids in which lyophilized samples (151.6 ± 0.9; 36.0 ± 16.2) resulted in a lower content than hot air-dried ones (200.8 ± 0.6, 62.8 ± 10.4). Regarding hot air drying, the temperature affected polyphenols content significantly ($p \leq 0.05$). Generally, hot air drying at 60 °C resulted in a lower content of polyphenols than hot air drying at 70 °C. However, in the case of rutin flavonoid (Figure 3.10B), the degradation was bigger at hot air drying 70 °C (195.2 ± 0.2) than at hot air drying 60 °C (206.4 ± 0.9). The overall trends observed in the ten specific polyphenols analysed were confirmed for the total phenol content (Figure 3.10D). Processing had a significant effect ($p \leq 0.05$) on the total phenol content and the effect was bigger in

hot air drying at 60 °C (0.243 ± 0.008), followed by hot air drying at 70 °C (0.279 ± 0.014) and lyophilization (0.4 ± 0.2). Total antiradical capacity (Figure 3.10D) also was affected in a significant way ($p \leq 0.05$) by processing. In DPPH results, there were not significant differences ($p \leq 0.05$) among the drying treatments. In ABTS results, there were significant differences ($p \leq 0.05$) among treatments resulting in lower antiradical capacity in lyophilized samples (0.21 ± 0.06) followed by hot air dried at 70 °C (0.67 ± 0.02) and hot air dried at 60 °C (0.72 ± 0.05).

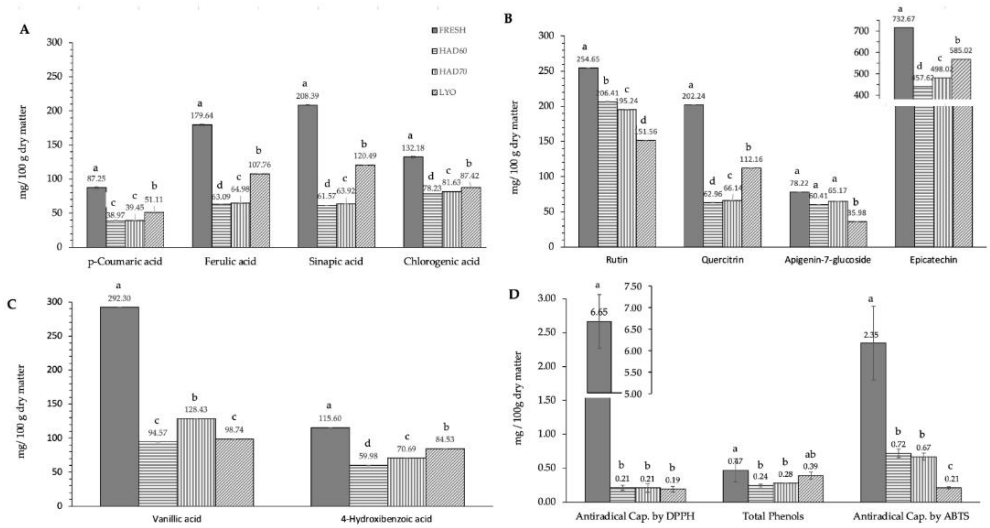


Figure 3.10. Effect of dehydration treatment on the polyphenols content and antiradical capacity. Phenolic compounds have been grouped according to their chemical structure A: Hydroxycinnamic acid derivatives (p-coumaric acid, sinapic acid, ferulic acid and chlorogenic acid), B: Flavonoids and derivatives (rutin, quercetin-3-glucoside, quercitrin, epicatechin and apigenin-7-glucoside), C: Hydroxybenzoic acid derivatives (vanillic acid, 4-hydroxybenzoic acid). Section D includes antiradical capacity determined by DPPH and ABTS methods and total phenols content. FRESH: Fresh almond bagasse, HAD60: Hot air dried powder at 60 °C, HAD70: Hot air dried powder at 70 °C, and LYO: Lyophilized powder. In section 2d: DPPH and ABTS results were expressed as mg Trolox/ 100 g dry matter; total phenols were expressed as mg gallic acid/ 100 g dry matter. Different superscripts letters mean statistically significant differences ($p \leq 0.05$).

3.2. *In vitro* gastrointestinal digestion.

The *in vitro* gastrointestinal digestion caused a significant effect ($p \leq 0.05$) in all the phenolic compounds determined. This effect was different depending on the considered polyphenol. Although evolution along simulated digestion was variable, the *in vitro* digestion resulted in a significant reduction of the specific polyphenol compounds. It was remarkable the absence of considered molecules after the colonic fermentation (Figures 3.11, 3.12 and 3.13).

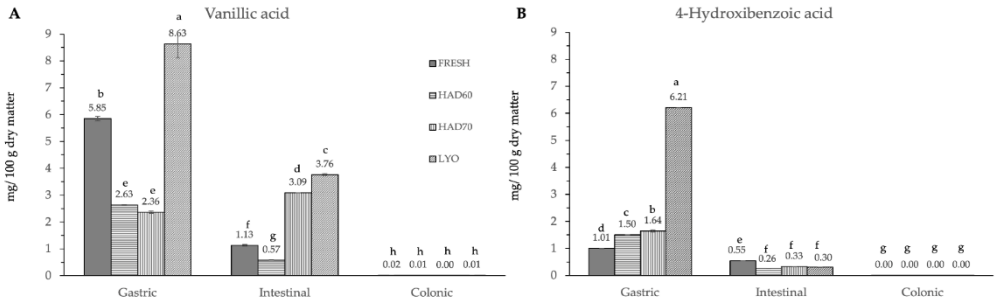


Figure 3.11. Hydroxybenzoic acid derivatives (A: vanillic acid, B: 4-hydroxybenzoic acid) content after gastric, intestinal, and colonic stages of *in vitro* gastrointestinal digestion. FRESH: Fresh almond bagasse, HAD60: Hot air dried powder at 60 °C, HAD70: Hot air dried powder at 70 °C, and LYO: Lyophilized powder. Different superscripts letters mean statistically significant differences ($p \leq 0.05$).

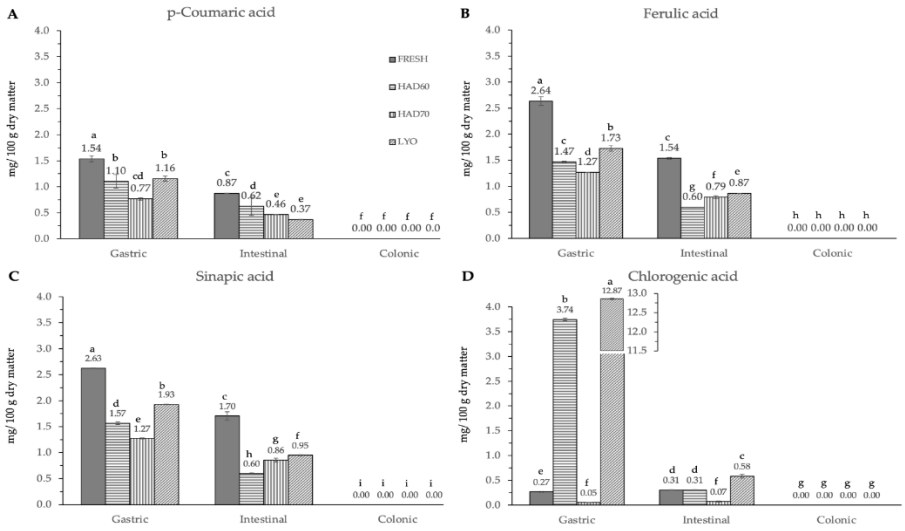


Figure 3.12. Hydroxycinnamic acid derivatives (A: p-coumaric acid, B: ferulic acid, C: sinapic acid and D: chlorogenic acid) content after gastric, intestinal, and colonic stages of *in vitro* gastrointestinal digestion. FRESH: Fresh almond bagasse, HAD60: Hot air dried powder at 60 °C, HAD70: Hot air dried powder at 70 °C, and LYO: Lyophilized powder. Different superscripts letters mean statistically significant differences ($p \leq 0.05$).

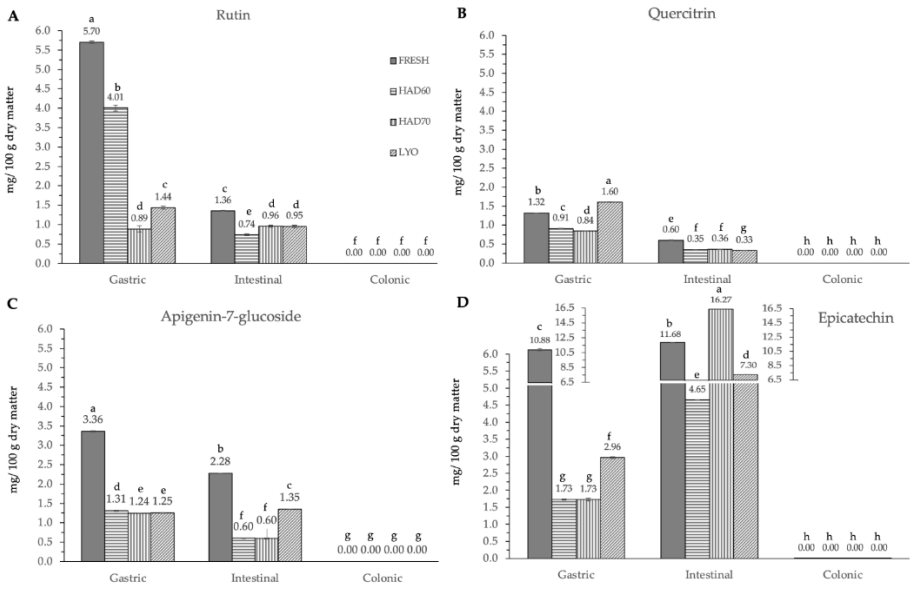


Figure 3.13. Flavonoids and derivatives ((A) Rutin, (B) Quercetin-3-glucoside, quercitrin, (C) Apigenin-7-glucoside and (D)Epicatechin) content after gastric, intestinal, and colonic stages of *in vitro* gastrointestinal digestion. FRESH: Fresh almond bagasse, HAD60: Hot air dried powder at 60 °C, HAD70: Hot air dried powder at 70 °C, and LYO: Lyophilized powder. Different superscripts letters mean statistically significant differences ($p \leq 0.05$).

In the gastric stage, the enzymes mixture and acidic conditions significantly reduced the content of most of the components. Specifically, for hydroxycinnamic acid derivatives (Figure 3.12) and flavonoids (Figure 3.13), the content determined was higher in fresh samples. However, in the case of chlorogenic acid (Figure 3.12D) and quercitrin (Figure 3.13B), the higher content was determined in lyophilised ones (12.87 ± 0.02). Hot air drying had, mostly, a similar effect on polyphenols determined regardless of the air temperature with the exception of chlorogenic acid. The intestinal enzyme mixture significantly reduced ($p \leq 0.05$) the content of polyphenols determined, except for epicatechin (Figure 3.13D). The content of epicatechin increased in a significant way ($p \leq 0.05$) after the intestinal stage in the samples after

all the treatments and in the fresh ones. Results are similar to those published by Curvas-Limón et al. [24] for the epicatechin from *Aloe Vera*. In our case, the increase was highest in hot air dried samples at 70 °C (16.27 ± 0.04), followed by lyophilised (7.30 ± 0.03), hot air dried at 60 °C (4.65 ± 0.02) and fresh ones (11.7 ± 0.2) (Figure 3.13D).

The significant ($p \leq 0.05$) and progressive increase observed in the total content of phenolic compounds and in both measurements of antiradical capacities after gastric, intestinal, and colonic stages (Figure 3.14). The results were different depending on the reagent used. The total phenolic compounds (Figure 3.14C) increased in a significant way ($p \leq 0.05$) at gastric, intestinal, and colonic stages. Although at gastric stage the total phenolic content of fresh samples was lowest (0.09 ± 0.01), it significantly increased ($p \leq 0.05$) at intestinal (2.30 ± 0.06) and colonic stages (5.5 ± 0.4). There were no significant differences ($p \leq 0.05$) among the three dehydration treatments regardless the digestion stage. In DPPH methodology (Figure 3.14A) the highest antiradical capacity ($p \leq 0.05$) was found in fresh samples (6.7 ± 0.6). The antiradical activity increased at gastric, intestinal, and colonic stages. There were no significant differences ($p \leq 0.05$) found among the values obtained in a gastrointestinal stage at three dehydration treatments. The same trend observed in the antiradical capacity values measured by the DPPH method and the total phenolic content are probably due to the higher sensitivity of both determinations to low hydrophilic compounds. In ABTS methodology he highest content ($p \leq 0.05$) was found at the intestinal stage in fresh samples (5.9 ± 0.2), decreasing again in the colonic stage (1.75 ± 0.08). Among the three dehydration treatments, the values obtained by lyophilized samples were significantly different ($p \leq 0.05$) and increased at each gastrointestinal stage (0.16 ± 0.05), (0.45 ± 0.02), (1.42 ± 0.62).

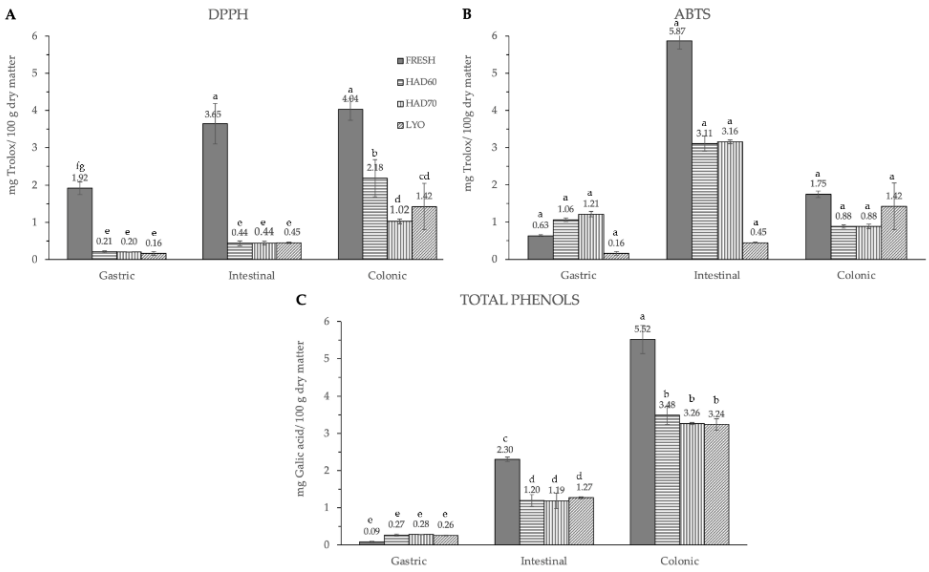


Figure 3.14. Antiradical capacity by DPPH (A), ABTS (B) methods and total phenols (C) after gastric, intestinal, and colonic stages of *in vitro* gastrointestinal digestion. FRESH: Fresh almond bagasse, HAD60: Hot air dried powder at 60 °C, HAD70: Hot air dried powder at 70 °C, and LYO: Lyophilized powder. DPPH and ABTS results were expressed as mg Trolox/ 100 g dry matter. Total phenols were expressed as mg gallic acid/ 100 g dry matter. Different superscripts letters mean statistically significant differences ($p \leq 0.05$).

3.3. Fermentative microbiota analysis

Sequencing of the 16S rRNA gene amplicons of the bacterial communities resulting from the *in vitro* fermentations of fresh almond bagasse, hot air dried powder at 60 °C (HAD60), hot air dried powder at 70 °C (HAD70), lyophilized powder (LYO), and controls were performed. The processing of raw reads (1,494,886) yields 1,173,186 sequences, averaging 86,284 genera per sample. The taxonomic assignment was performed at the genus level.

Canonical correspondence analysis (CCA) and the adonis test (Figure 3.15) revealed a significant difference in the microbial community structures. As no

significant differences were detected in the fermentative microbiota of air dried powder at 60 °C (HAD60) and air dried powder at 70 °C (HAD70) (p-value = 0.4), they were considered as a single air drying treatment for the analysis. The CCA showed the first axis, which explained 55.60% and 60.81% of the variability (Figure 3.15), and separated substrate-fermentation microbiota and controls (fermentations without substrates). The control overgrowth would be due to residual nutrients in the faecal samples used as inoculum. The second axis, explaining 23.84% and 26.25% of the variability, separated the bacterial community that grew on fresh bagasse from that which fermented the heat-treated (HAD) or lyophilized one (LYO).

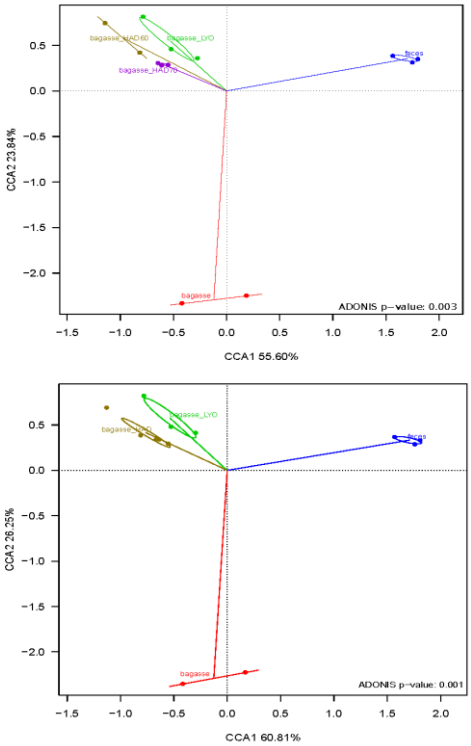


Figure 3.15. Canonical correspondence analyses (CCAs) at genus level of the bacterial community after fermentations. Bag asse: fresh almond bagasse; bagasse HAD60: hot air dried powder at 60 °C; bagasse HAD70: hot air dried powder at 70 °C; bagasse LYO: lyophilized powder; faeces: controls.

To assess which bacteria were preferentially growing on air-dried, lyophilized, and fresh bagasse, a pairwaise comparisons with the faeces fermentations as controls using ANCOMBC package (Figure 3.16 and Figure S3.1) was performed. Thus, it was found 8 genera that were differentially abundant after the bagasse fermentations, being *Butyrivibrio* from Lachnospiraceae family the genus that presented the greatest increase in abundance in comparison with faeces fermentations (Figure S3.1 and Table S3.1). The other bacterial groups found at higher abundance in bagasse fermentations were *Eubacterium eligens* group, *Ruminococcus torques* group, *Fusicatenibacter*, *Dorea* from Lachnospiraceae; *Romboutsia* from Peptostreptococcaceae; *Butyricicoccus* from Butyricicoccaceae and *Phascolarctobacterium* from Acidaminococcaceae (Figure S3.1 and Table S3.1). Both dehydration techniques, air-dried and freeze-dried, favour the growth of *Allisonella* and *Dialister* belonging to Veillonellaceae family as well as the pathobiont *Cloacibacillus* from Synergistaceae. Additionally, *Lachnospira* and *Clostridium sensu stricto* 1 were more abundant after air-dried and fresh bagasse fermentations. Likewise, *Holdemanella* presented higher abundance after air-dried bagasse fermentation. Three Enterobacteriaceae genera and Defluviitaleaceae UCG-011 presented more abundance after fresh bagasse fermentation. In order to investigate the effect on the microbiota composition of the dehydration treatment, it was applied ANCOMBC analysis for the pairwaise comparisons between fresh bagasse, bagasse_HAD and bagasse_LYO (Figure 3.16B, Figure S3.2 and Table S3.2). Both heat-treated and freeze-dried bagasse fermentations resulted in a microbiota with an increased abundance of *Allisonella* (bagasse_HAD, q=0.0002; bagasse_LYO, q=4.1547E-015), *Ruminococcus* (bagasse_HAD, q=0.0116; bagasse_LYO, q=0.0201) and *Erysipelotrichaceae UCG-003* (bagasse_HAD, q=0.0002; bagasse_LYO, q=0.2636) genera than that of the fresh bagasse. Moreover, heat treatment of the bagasse gave

rise to an increase in the abundance of several SCFA-producer genera such as *Eubacterium* (p=0.0495, q=0.1424), *Coprococcus* (p=0.0014, q=0.0118), *Ruminococcus* (p=0.0194, q=0.0835), *Agathobacter* (p=0.0004, q=0.005) or *Subdoligranulum* (p=0.0135, q=0.0619) after the colonic fermentation.

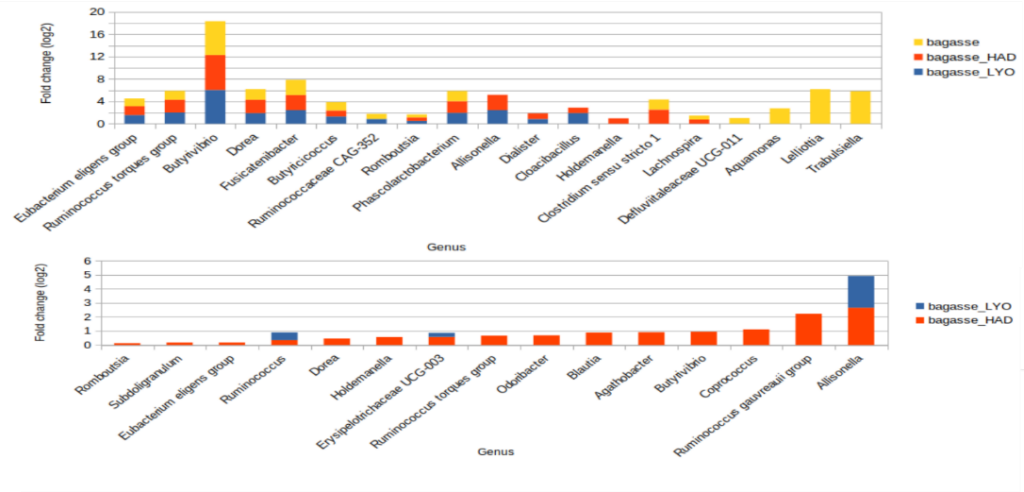


Figure 3.16. Barplot of fold-changes of bacterial genera that are significative increased in a) pairwaise comparisons with faeces fermentations and in b) pairwaise comparisons with the fresh bagasse fermentations. Freeze-dried bagasse (bagasse_LYO), air-dried bagasse (bagasse_HAD) and fresh bagasse (bagasse).

4. Discussion

4.1. Effect of processing on antiradical capacity and phenol content

The polyphenol composition of almond (*Prunus dulcis*) is significantly affected by harvest time, environmental factors, agriculture practices, ripening and variety [25]. As presented above, catechin was also the most abundant polyphenol found in almond kernels by previous studies [26], [27], [28]. However, data found in literature referred to polyphenols content in almond kernel were variable probably due not only to agronomic conditions, maturity stage and variety but also to the extraction

solvent and methodology [25]. In almond bagasse, water extraction from the kernel could solubilize polyphenolic components to varying degrees depending on their hydrophilicity. For a more accurate investigation the compounds present in fresh and/or processed bagasse should be identified by NMR spectroscopy and compared to those found in the integral product. Furthermore, EPR methodology could be used to determine both oxidative stress and antioxidant capacity. These are the main limitation of this study.

The bagasse had a high humidity and water activity, and needed to be stabilized to prevent degradation and prolong shelf-life so that it could be recovered and reused. Hot air drying at two temperatures and lyophilization were carried out. In both dehydration treatments, the reduction in polyphenolic compounds could be due to the chemical and enzymatic reactions induced in the product by the temperature or be facilitated by structural changes and interactions that occurred in the samples. The main structural changes induced by dehydration treatments were cellular turgor loss, alteration of the middle lamella, altered cell wall strength, changes in the volume fractions of gas and liquid, as well as changes in the size and shape of the samples [29]. They all could facilitate the interaction between enzymes and the wide variety of bioactive components, such as polyphenols and other macromolecules acting as affective activators of oxidation reactions and located in the different cell structures. For example, George et al. [30] showed that low concentrations of the free fatty acids such as oleic acid, linoleic acid, and arachidonic acid were effective activators of epicatechin oxidation *in vitro*, suggesting that these endogenous free fatty acids may play a role in the polyphenol oxidase-mediated browning of avocado fruit in vivo. In lyophilization, the freezing and sublimation of water degraded cellular structure and promoted the formation of a porous structure. However, the absence of high temperatures and oxygen could reduce degradation.

In hot air-drying treatments, structural damage and oxygen presence at a high temperature resulted in a higher degradation. Nevertheless, the difference between 60 and 70 °C could be decisive in the inactivation of enzymes involved in some of the degradation reactions. Regarding the interaction with other molecules, dehydration has been shown to increase polyphenolic compounds, despite some degradation, because it may improve extraction and lead to a greater release of these compounds [10]. In almond bagasse, the macronutrient composition, consisting mainly of fibre and fat, could interact with the polyphenols and prevent their release after processing.

The differences found between the antiradical capacity measurements were probably due to the differences between the bioactive compounds and the radical affinity of ABTS or DPPH. In fact, the DPPH method was found to be more sensitive to hydrophobic antiradicals, while the ABTS method was found to be more sensitive to hydrophilic antiradicals [31].

4.2. *In Vitro* Gastrointestinal Digestion and Colonic Fermentation

As described above, there is generally a progressive increase in some polyphenol contents and antiradical capacities as gastrointestinal digestion progresses. A possible hypothesis that could explain the results obtained would be based on the interactions established among bioactive compounds and macromolecules. Bioactive compounds can be free or bonded. Structural changes and oxidation reactions induced by dehydration treatment would have a stronger effect in free compounds. Thus, the mixture of enzymes and pH variations at gastric stages affected only the free bioactive compounds of fresh samples but not those remaining bound in the treated ones. However, the mixture of enzymes and pH variations at the intestinal stages could affect the interactions of the bioactive compounds,

promoting the release of these treated or fresh compounds. It seems that there was a progressive structural degradation that started to release the bioactive compounds after initial degradation. At the colon stage, the gut microbes metabolized polyphenols leading to smaller size metabolites that were more bioavailable than the original ones [32]. The resulting metabolites showed high bioactivity [33]. This effect can be explained considering the reaction sequences suffered by the polyphenols in the gut, promoted by the enzymes produced by microbiota. The sequence followed to convert polyphenols into smaller metabolites is well described in the literature [32]. The presence of an ester moiety in the hydroxycinnamic acid reduced their intestinal absorption and reached the colon where the gut microbial esterase performed deconjugation and released the free acid from esters. Hydroxycinnamic acid metabolism by the gut microbiota involved many other biochemical transformations such as (de)hydrogenations, (de)methylations, (de)hydroxylations, b-oxidations, etc., leading to diverse metabolites detected in human plasma and urine in individuals with a diet rich in hydroxycinnamic acids [34]. Glycosidic deconjugation to release aglycones, the hydrogenation of the double bond, opening of the C-ring, and catechol-dehydroxylations are the main metabolic pathways of flavonoids in the colon to release smaller molecules with the capability to be absorbed [32].

4.3. Fermentative Microbiota Analysis

In a previous work [10], it was indicated that the substrates affected the microbial community growing during the fermentation. Thus, the bacterial communities that grew on both fresh and treated substrates presented a significantly different structure to the residual bacterial population of the fermentation controls. Additionally, the dehydration of the bagasse has a clear effect on the composition of

the *in vitro* fermentative microbiota. Interestingly, the air-dried technique benefits the growth of several SCFA-producer genera. The great abundance of the Butyrivibrio genus detected after the fermentation of the three bagasses, fresh, heat-treated, and freeze-dried, suggested that the composition of the almond substrate favours its growth. Different species of Butyrivibrio are involved in plant polysaccharide breakdown and butyrate production, a short-chain fatty acid that has beneficial health effects for the host [35]. Moreover, the genera Eubacterium, Fusicatenibacter, Romboutsia, Phascolarctobacterium, and Ruminococcus, which presented high abundance after bagasse fermentations, have been also described as fibre-degraders and butyrate-producers [36,37]. Based on comparative genome analysis, Gerritsen et al. [38] showed that the genus Romboutsia encode a versatile array of carbohydrate metabolic capabilities, respectively, carbohydrate utilization producing formate, acetate, lactate, and butyrate. Phascolarctobacterium has been described as an acetate and propionate producer and is positively associated with positive mood in humans [39]. Moreover, Phascolarctobacterium faecium has been related to the tolerance of metformin in type 2 diabetes patients [40]. Likewise, Holdemanella, Allisonella, and Dialister presented with higher abundance after dehydrated-bagasse fermentations. Valles-Colomer et al. [41] described Dialister and Coprococcus as neuroactive genera that are depleted in depression. A study in mice showed that Holdemanella biformis ameliorates hyperglycemia, improves oral glucose tolerance, and restores gluconeogenesis and insulin signaling in the livers of obese mice [42]. Thus, the colonic fermentation of dehydrated bagasses, specially air-dried bagasse, promotes the growth of commensal bacteria described as beneficial to human health.

5. Conclusions

Almond bagasse is an interesting by-product, not only for its fibre content, but also for its richness in polyphenols. Its stabilization via a controlled dehydration treatment combined with milling is an alternative for producing functional powdered ingredients for the food industry. Air drying at 60 and 70 °C and lyophilization reduced the content of all the specific polyphenols analysed. The retained content was very variable depending on the polyphenol considered and the treatment applied. There was no defined trend. The maximum retention (83.3%) was for apigenin -7- glucoside after air drying at 70 °C and the minimum (29.5%) was for sinapic acid after air drying at 60 °C.

In terms of antiradical scavenging activities and total phenolics, each of the treatments considered significantly reduced the levels. However, *in vitro* digestion and the intestinal and colonic phases, although still reducing the specific polyphenol content in all the powdered products, made it possible to recover much of the antiradical capacity from the fresh product.

The fermentation of almond bagasse, both fresh and dehydrated, promoted the growth of Butyrivibrio, which is a genus described as a fibre degrader and butyrate producer. Thus, the increase in the Butyrivibrio genus could have implications for human health. Moreover, the dehydration techniques influenced the composition of the *in vitro* resulting microbial community, with the air-dried method giving rise to a greater abundance of SCFA-producer genera.

It would be necessary to follow up on the polyphenol biotransformation into smaller molecules/secondary metabolites to identify and describe in detail the increase in the antiradical activity and the total phenols, and how this relates to gut

microbiota. Moreover, understanding the role of other macronutrients in the bioaccessibility of polyphenols, in their degradation or biotransformation, is of vital importance to provide relevant and holistic information about the product as well as a detailed effect of dehydration treatments and the digestion process.

Supplementary Materials

Table S3.1. Pairwise comparisons between faeces and bagasse_LYO, bagasse_HAD or fresh bagasse.

	Bagasse_LYO		Bagasse_HAD		Fresh Bagasse	
Genus	p-value	q-value	p-value	q-value	p-value	q-value
Eubacterium eligens group	1,0932E-06	5,9345E-06	7,3608E-10	5,0804E-09	0,0023	0,0107
Ruminococcus torques group	8,2754E-07	4,8379E-06	2,5295E-08	1,4093E-07	0,0021	0,0107
Butyrivibrio	8,8966E-38	3,3807E-36	2,0238E-57	3,1572E-56	1,2626E-14	3,3249E-13
Dorea	0,0001	0,0005	4,3984E-08	2,2872E-07	0,0028	0,0117
Fusicatenibacter	0,0057	0,0145	0,0001	0,0003	0,0195	0,0617
Butyricicoccus	0,0082	0,0195	0,0062	0,0110	0,0086	0,0325
Ruminococcaceae CAG-352	0,0014	0,0046	ns	ns	0,0062	0,0245
Romboutsia	0,0363	0,0708	0,0007	0,0016	0,0377	0,1103
Phascolarctobacterium	6,5179E-14	5,5040E-13	1,9645E-07	9,5767E-07	7,8464E-05	0,0006
Allisonella	0,0033	0,0093	0,0053	0,0096	nd	ns
Dialister	0,0447	0,0850	0,0101	0,0172	nd	ns
Cloacibacillus	0,0173	0,0364	ns	ns	nd	ns
Holdemanella	ns	ns	0,0028	0,0055	nd	ns
Clostridium sensu stricto 1	ns	ns	4,4886E-05	1,2504E-04	0,0125	0,0429
Lachnospira	ns	ns	0,0176	0,0286	0,0342	0,1039
Defluviitaleaceae UCG-011	ns	ns	ns	ns	0,0114	0,0409
Aquamonas	ns	ns	ns	ns	3,5344E-07	3,9169E-06
Lelliottia	ns	ns	ns	ns	1,2359E-42	9,7634E-41
Trabulsiella	ns	ns	ns	ns	3,5344E-07	3,9169E-06

Table S3.2. Pairwise comparisons between fresh bagasse and bagasse_HAD or bagasse_LYO.

	Bagasse_LYO		Bagasse_HAD	
Genus	p-value	q-value	p-value	q-value
Romboutsia			0,0302	0,1041
Subdoligranulum			0,0135	0,0619
Eubacterium eligens group			0,0495	0,1424
Ruminococcus	0,0015	0,0201	0,0012	0,0116
Dorea			0,0047	0,0335
Holdemanella			0,0073	0,0419
Erysipelotrichaceae UCG-003	0,0280	0,2636	6,7429E-06	0,0002
Ruminococcus torques group			0,0194	0,0835
Odoribacter			0,0355	0,1112
Blautia			0,0064	0,0401
Agathobacter			0,0004	0,0050
Butyrivibrio			0,0339	0,1112
Coprococcus			0,0014	0,0118
Ruminococcus gauvreauii group			0,0084	0,0446
Allisonella	2,5180E-16	4,1547E-15	1,1345E-05	0,0002

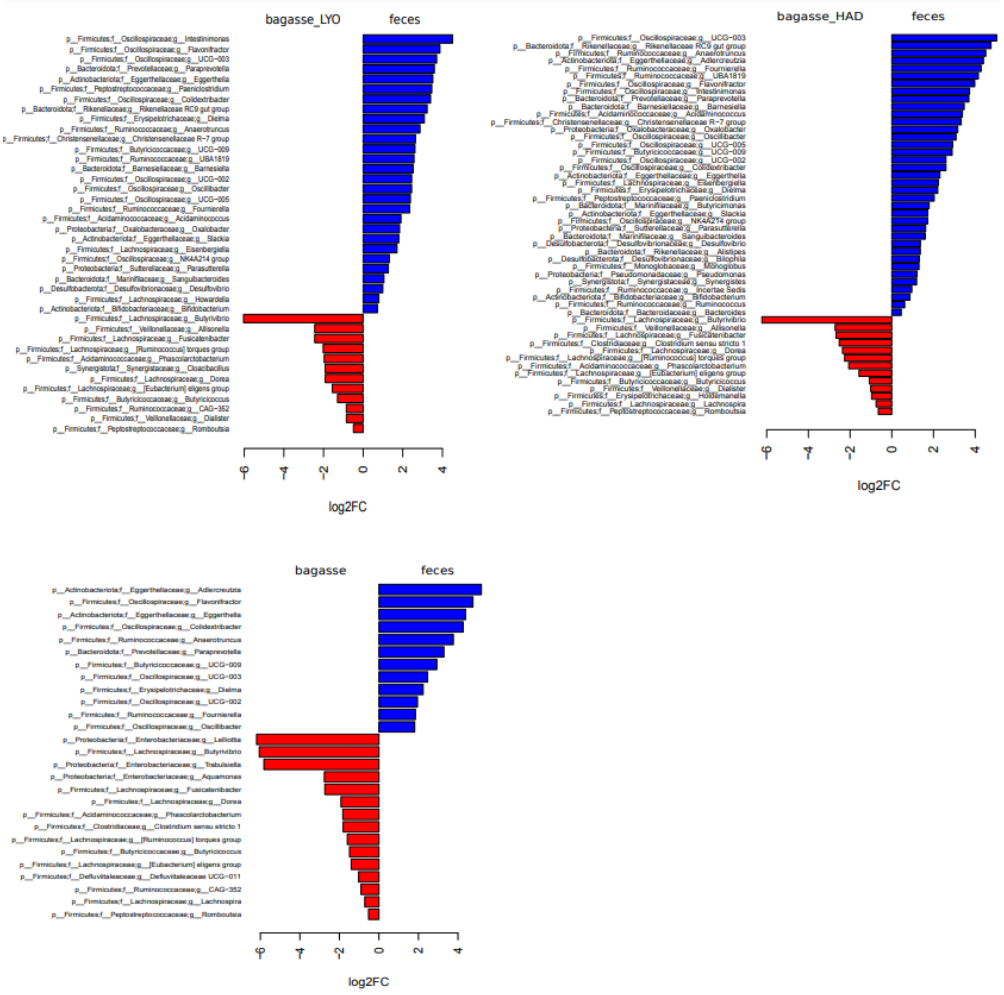


Figure S3.1: Barplot of fold-changes in relative abundance of bacterial genera between faeces fermentation and freeze-dried bagasse (bagasse_LYO), air-dried bagasse (bagasse_HAD) and fresh bagasse (bagasse) fermentations. Fold-changes are represented as log2(FC) and negative values indicate enriched genera in bagasse_LYO, bagasse_HAD and bagasse fermentations.

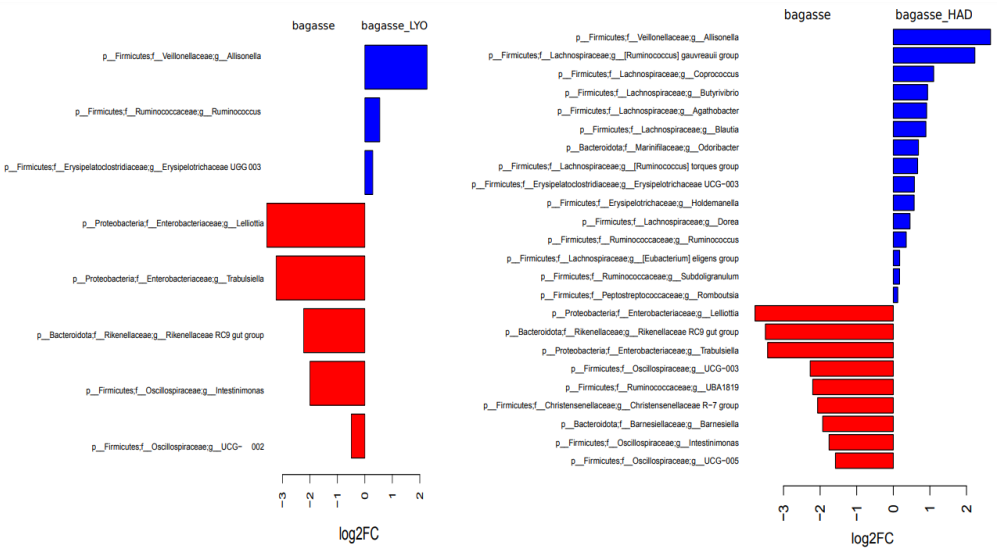


Figure S3.2. Barplot of fold-changes in relative abundance of bacterial genera between fresh bagasse (bagasse) fermentation and freeze-dried bagasse (bagasse_LYO) and air-dried bagasse fermentations. Fold-changes are represented as log2(FC) and negative values indicate enriched genera in fresh bagasse fermentation.

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ARTÍCULO 5: Effect of dehydration on the production of Short and Branched Chain Fatty Acids (SCFA and BCFA) in almond bagasse during colonic fermentation *in vitro*.

1. Introduction

The human colon harbours more than 100 trillion microorganisms belonging to over 1000 different microbial species. Among this microbial community, the most predominant bacteria are Firmicutes (constituting 65%), followed by Bacteroidetes (representing 25%), Actinobacteria (around 5%), and Proteobacteria (approximately 8%) [1]. This microbial population in the colon plays a fundamental role in various metabolic and immune functions, and its composition can significantly influence the health of the host individual [2]. Bacteria resident in the colon are highly engineered to break down dietary fibre. Recent research has revealed that the composition of the microbial community can be influenced by the presence of dietary fibre in the diet. The ability of microorganisms to break down specific types of fibre depends on whether their genomes contain the enzymes necessary for this task, as well as related proteins [3].

Fermentation is an important process in the large intestine, or colon, in which anaerobic bacteria break down carbohydrates into short-chain fatty acids (SCFA), gases such as hydrogen, methane and carbon dioxide, along with other metabolites. Although most SCFA (around 90-95%) consist of acetate, propionate and butyrate, smaller proportions of valerate, hexanoate and branched-chain fatty acids (BCFA), such as isobutyrate and isovalerate, are also found. The BCFA are produced because of the breakdown of proteins rather than carbohydrates [4,5]. Importantly, butyrate is the main source of energy for colon cells and has been shown to have a preventive effect on the development of colon cancer [6]. The production of short-chain fatty acids (SCFA) by anaerobic fermentation is conditioned by the type of dietary fibre serving as substrate, as reported in previous studies [7]. As it is shown in Table 3.11, the nature and structure of food-derived fibres can influence the composition and

activity of microorganisms, as well as the amounts and proportions of SCFA metabolites produced. For example, research has shown that xylan, β-glucan, xylo-oligosaccharides and some fibres such as cassava pulp promote the generation of butyric and acetic acid [8–10], also lignocelluloses generate higher amounts of acetic and propionic acid [11]. However, protein-rich products such as bovine serum, casein and soy protein promote the production of branched-chain fatty acids such as isobutyrate and isovaleric [12]. In the last decade, next generation sequencing techniques, especially those based on 16S rRNA analysis, have revolutionized research on how microbial communities in human faeces respond to dietary fibre interventions. It has been extensively investigated in *in vitro* studies. Overall, pectin, resistant starch, inulin and fructooligosaccharides have been found to selectively promote the growth of Bifidobacteria. Furthermore, a positive correlation has been identified between propionic acid production and the presence of Bacteroides, while butyric acid production is positively related to the presence of Ruminococcaceae and Faecalibacterium [13,14].

Table 3.11. Changes in SCFA, BCFA and microbiota after *in vitro* batch fermentation performance of various dietary fibres using human faecal inocula [15].

Dietary fibre	SCFA changes		Microbiota composition changes		References
	Increase	Decrease	Increase	Decrease	
Rice and maize (resistant starch type 2)	Acetate	Butyrate	*Bifidobacterium*	*Blautia, Dorea, Bacteroides*	[8,13]
Red wheat and rice (resistant starch type 3)	Acetite, butyrate	-	*Bifidobacterium*	-	[8,16,17]
Corn and potato (resistant starch type 4)	Acetate, butyrate	-	*Bifidobacteria*	-	[18,19]
Rice bran (arabinoxylan)	Acetate, butyrate	-	*Bacteroides, Coprococcus, F aecalibacterium*	-	[13,20]
Tamarind (xyloglucan)	Propionate , butyrate		*Lachnospiraceae, Bacteroides*	-	[21]
Agave (inulin)	Acetate, butyrate	Propionate	*Bifidobacterium, Catenibacterium, Collinsella*	*Bacteroides, Dorea*	[13]
Pectin	Acetate, butyrate	Propionate	*Bifidobacterium, Lachnospira, Clostridiu, Sutterella*	*Bacteroides, Dorea, Parabacteroides*	[13,17,22,23]
Guar gum	Acetate, butyrate	Propionate	*Roseburia*	-	[13,17]
Konjac (glucomannan)	Acetate, Propionate	Butyrate	-	-	[17]
Barley and oat (β-glucan)	Acetate, butyrate	-	*Coprobacillus, Lactobacillus, Enterococcus*	*Dorea*	[13,17,24]
Cellulose	Acetate, butyrate	-	*Bacteroides sp, Ruminococcus sp, Enterococcus sp*	-	[17,25]
Fructo-oligosaccharides	Acetate, butyrate	-	*Lactobacilli, Prevotella*	-	[7,17,26]
Galacto-oligosaccharides	Propionate , butyrate	-	*Bifidobacteria*	-	[7]
Bovine serum	Isovaleric, isobutyrate	-	*Megasphaera elsdenii*	-	[12]
Casein	Isovaleric, isobutyrate	-	*Megasphaera elsdenii*	-	[12]
Soya protein	Isovaleric, isobutyrate	-	*Bifidobacteria, Lactobacilli*	-	[27]
Cassava pulp	Acetate, butyrate	Valeric	*Bifidobacteria, Lactobacilli*	-	[10]
Lignocellulose	Acetate, propionate	-	*Bifidobacteria, Lactobacilli*	-	[11]
Xylo-oligosaccharides	Butyrate	-	*Blautia*	-	[9]
Oat hulls	-	Acetate, propionate, butyrate, isobutyrate, isovaleric	*Bifidobacteria, Lactobacilli, Enterobacteriaceae*	-	[6]

Mass spectrometry (MS), in combination with separation techniques such as gas chromatography (GC) or liquid chromatography (LC), is commonly used in the metabolic analysis of biological samples due to its high sensitivity and selectivity. In the case of SCFA and BCFA quantification, LC-MS excels in requiring minimal sample preparation. However, in LC-MS accurate quantification of SCFA without the need for chemical derivatization requires the use of demanding experimental conditions, such as highly acidic mobile phases. Moreover, the hydrophilicity of SCFA can lead to additional problems, such as poor chromatographic separation and insufficient ionization. To address these challenges, various chemical derivatization methods have been developed for the quantification of SCFA and BCAA with LC-MS. These methods often involve long reaction times or specific reaction conditions [28,29], For this reason, GC remains the most widely used technique due to the volatility of SCFAs and the high sensitivity, good resolution and relatively low cost of the technique and sample pre-treatment. GC can be coupled to a flame ionization detector (FID) [30–32]. The primary objective of this study was to determine the effect of dehydration method and air drying temperature on the production of short chain fatty acids (SCFAs) during *in vitro* colonic fermentation of almond bagasse. Furthermore, it will attempt to clarify the relationship between substrate, microbiome and SCFAs.

2. Material and methods

2.1. Process for obtaining almond bagasse and almond bagasse powder.

Almonds purchased from a local supermarket were mixed with tap water in a proportion 1/9 (w/w). Using a household food processor (Thermomix®, Vorwerk, Spain) operating at 10,000 revolutions per minute for 20 seconds, the almonds were finely ground. Subsequently, the resulting ground mixture was passed through a stainless steel sieve with a mesh size of 500 µm. The residue, referred to as almond

bagasse, was subjected to drying in a convective dryer (Pol-eko Aparatura, Katowice, Poland) with an airflow at a rate of 10 m/s at temperatures of 60 °C and 70 °C (HAD) for 10 and 7 h, respectively, until its water activity (aw) dropped below 0.3. Furthermore, a freeze dryer (Telstar, Lioalta-g) was employed to produce freeze-dried product (LYO) from the almond bagasse, which had been previously frozen at -40 °C for 24 hours. Following the drying process, the dried product was subjected to grinding using a food processor (Thermomix®, Vorwerk, Spain) at 4,000 rpm for 20 seconds, with intervals of 5 seconds, and then at 10,000 rpm for 20 seconds, with intervals of 5 seconds, to obtain almond bagasse powder.

2.2. *In Vitro* gastrointestinal digestion.

For *in vitro* gastrointestinal digestion of almond bagasse powders, the method described by Minekus et al. [33] was followed. Oral, gastric, and intestinal stages were followed by *in vitro* batched colonic fermentation. In gastrointestinal digestion stages, a volume ratio of 1:1 (v/v) of the combined phases was maintained. First, 5 g of the sample were added to a 50 mL Falcon tube. After that, 5 mL of simulated liquid saliva was introduced, and the pH was adjusted to 7. After a period of 5 minutes, 10 mL of the pepsin-containing gastric solution was added, pH was adjusted to 3 and left to incubate for 2 h. In the intestinal stage, the gastric reaction was stopped by increasing the pH. Subsequently, 20 mL of the intestinal solution, which included pancreatin trypsin and bile, was added, and the pH was adjusted to a neutral level. After two hours of reaction, the digestion process was completed by lowering the pH to deactivate the enzymes. 1 N HCl was used to adjust the pH in the gastric stage and 1 N NaOH was used in the intestinal stage.

2.3. *In vitro* colonic fermentation.

Faecal samples were obtained from healthy donors who had not taken antibiotics, prebiotics or probiotics in the three months prior to the study. The study was conducted according to the guidelines of the Declaration of Helsinki, and participants gave informed consent before participating in the research. Donor faecal samples were pooled to reduce day-to-day variability within individuals, and this pooling was used to create a 10% (w/v) inoculum concentration. After subjecting the different samples (fresh bagasse (bagasse), hot air-dried powder at 60 °C (HAD60), hot air-dried powder at 70 °C (HAD70), freeze-dried powder (LYO)) to *in vitro* gastrointestinal digestion, the solid residue (fraction not available for intestinal absorption) remaining after removal of the supernatant plus 10% of the digestion supernatant, was used as substrate at a concentration of 1% (w/v) for colonic fermentation. Colonic fermentation was performed in triplicate as described by Bas-Bellver et al. [34]. In addition, a control fermentation was performed without substrate. Samples were taken from the fermenters at baseline (t = 0 h) and after 24 h for further analysis. All incubation and processing procedures were carried out under anaerobic conditions, using an anaerobic flask or an anaerobic chamber.

2.4. Extraction of short-chain fatty acids

For SCFA extraction, the protocol proposed by Tallarico-Adorno et al. [35] was followed. 500 µL of sample was mixed with 125 µL of a 9.2 N H_2S0_4 solution in a 2 mL Eppendorf tube. Then, a small amount of NaCl was added using the end of a glass Pasteur pipette. Afterwards, 100 µL of the internal standard (IS) was added which was prepared by mixing 1.5 mL of methylhexanoic acid with 98.5 mL of 0.1 N NaOH and made up to 200 mL with distilled water. Subsequently, 500 µL of diethyl ether was added to the Eppendorf tube, immediately closed and vortexed for 1 min. The

sample was then centrifuged at 300 xg for 3 min and the supernatant was transferred to chromatography vials. For SCFA analysis, a gas chromatograph coupled with a flame detector Agilent GC6890B-5977B GC FID (Agilent, Santa Clara, CA, EE. UU) with a multipurpose sampler with a SUPELCOWAX™ 10 Capillary GC Column (30 m × 0.25 mm × 0.25 µm, Merck, Rahway, NJ, USA) was used. The oven temperature was kept constant at 240 °C, with a flow of 35 mL/min of H_2, a flow of 250 mL/min of air and a flow of 40 mL/min of N_2 auxiliary gas. In addition, a temperature gradient was applied in the oven, starting at 90 °C and increasing to 240 °C, with an increase of 10 °C per minute, using the splitness technique. Analytical calibration curves were generated to measure the amount of the volatile acids of interest: acetic acid (AA), propanoic acid (PA), butyric acid (BA), valeric acid (VA), isovaleric acid (IVA) and isobutyric acid (IBA), ranging from 0 to 130 ppm. Results were reported in mg per g of sample.

3. Results

Figure 3.17 and Figure 3.18 shows the amount and diversity of total fatty acids, including SCFA and BCFA, and the ratio SCFA/BCFA after the *in vitro* batched colonic fermentation of the digested air-dried almond bagasse (HAD60, HAD70) and the lyophilised one. Although there is a high variability between individuals (inoculum 1-4), the air-dried samples produced the highest amount of total fatty acids (SCFA plus BCFA) in three of the four individuals, regardless of air temperature. 70 °C was the temperature that resulted in the highest SCFA production in 75% of the cases and the highest BCFA production in 50% of the cases. The SCFA/BCFA ratio resulted in a value >1 in the 41.7% of the cases mainly associated with freeze-dried samples; equal to 1 in the 16.7% of the cases regardless the dehydration method; and <1 in 33.3% of the cases mainly associated with air drying at 60 °C. The presence of a significant percentage of fibre (around 50%), which is the substrate used by colonic bacteria for

the production of SCFAs, and amino acids and/or proteins (around 16%) used for the production of BCFAs, justifies these results. However, the protein content is significantly lower than the fibre content, which should perhaps lead to a higher SCFA/BFA ratio. Despite this and considering that the large intestine is a highly proteolytic environment [36,37], almond bagasse could be an optimal substrate to drive the generation of branched-chain fatty acids (BCFA). This is evidenced by similar results obtained in products with a high protein content, such as soy protein, bovine whey, and casein, which promoted BFCA production and significantly reduced SCFA production [27,38]. On the other hand, a lower value than expected for SCFA could be attributed to the fact that as the fermentation phase progresses, the activity of saccharolytic bacteria decreases, leading to a reduction in SCFA concentration [27]. This includes a variety of bacteria, such as Eubacteria, Megasphaera Elsdenii, xaccharolytic bacteroids, asaccharolytic bacteroids and a variety of Gram-positive anaerobic cocci [38,39]. According to Duarte et al. [40], certain bacterial genera such as Firmicutes, Clostridium, Bacteroides and Ruminococcus predominate in the *in vitro* colonic fermentation of dehydrated almond bagasse. These bacterial genera are noted for their ability to carry out amino acid fermentation, which, in turn, stimulates the production of branched-chain fatty acids (BCFA). Although carbohydrate fermentation constitutes the main source of energy for the gut microbiota, microorganisms can turn to other sources when carbohydrates in the colon are depleted or unavailable [41]. These alternatives may include the use of proteins and amino acids. Moreover, although to a lesser extent there was an increase in bacilli after fermentation of almond bagasse powders, recent research [42,43] showed that, in addition to carbohydrates, certain additional compounds, such as anthocyanins, flavanols, proteins and lipids, can also stimulate the development of lactobacilli during the colonic fermentation process. Branched-chain fatty acids (BCFA) have a

fundamental role in the functioning of the nervous system by facilitating the transmission of signals between neurons [44]. They also play an essential role in regulating the secretion and absorption of electrolytes, thus contributing to the maintenance of balance in this process.

According to the study carried out by Duarte et al. [40], it was found that dehydration of almond bagasse promoted the growth of Butyrivibrio, a bacterium known for its ability to break down fibres and produce butyrate. This finding suggests that an increase in the presence of Butyrivibrio could have potential benefits for human health. Although no significant differences in butyric acid production were observed between the different dehydration methods, Duartechanges in microbial community composition were noted compared to fresh almond bagasse [45]. Fermentation of almond bagasse powders also promoted the production of valeric acid. According to Rasmussen et al. [4] this acid can also be generated from the fermentation of amino acids.

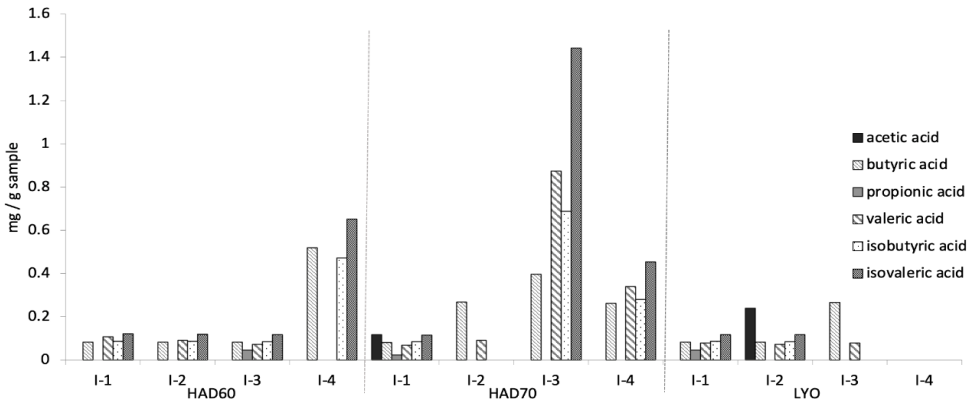

Figure 3.17. Fatty acids generated (mg/ g sample) after *in vitro* colonic fermentation of hot air-dried 60 °C (HAD60), 70 °C (HAD70) and freeze-dried (LYO) almond bagasse powder. I-1, I-2, I-3 and I-4 correspond to inoculums from four different individuals.

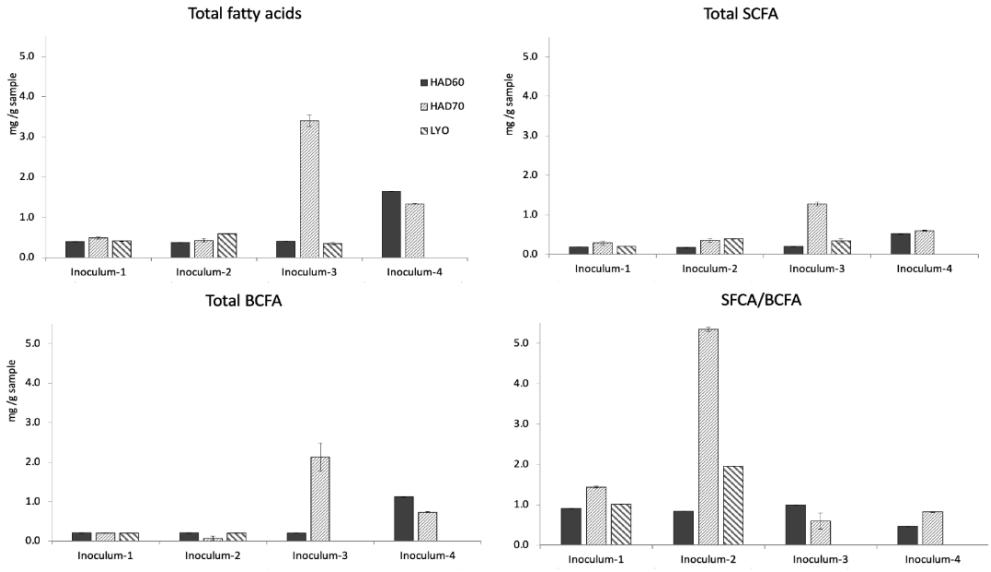

Figure 3.18. Amount and diversity of total fatty acids, including SCFA and BCFA, and the ratio SCFA/BCFA after the *in vitro* batched colonic fermentation of the digested air-dried almond bagasse (HAD60, HAD70) and the freeze-dried. Mean ± standard deviation of three repetitions.

4. Conclusions and final remarks

Almond bagasse could be an optimal substrate to drive the generation SCFA and BCFA. Butyric acid was the most abundant SCFA and valeric and isovaleric acids were the most abundant BCFA. However, it is important to note that there is a high variability among individuals and it will be recommendable to carry out the study with a larger population or with groups with specific nutritional needs.

Regarding drying method and drying temperature, it seems that hot air drying leads to higher total fatty acid production, however the results may be biased by the high variability between individuals.

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CONCLUSIONES CAPÍTULO II

El elevado contenido en fibra y polifenoles convierten el bagazo de almendra en un valioso subproducto con potencial para ser utilizado por la industria alimentaria como un producto en polvo estabilizado mediante deshidratación y molienda.

Los procesos de secado por aire caliente a 60 y 70 °C y liofilización afectaron el perfil de compuestos polifenólicos específicos, mostrando contenidos variables según el tratamiento aplicado. En cuanto a las capacidades antirradicales (ABTS y DPPH) y los fenoles totales, la digestión gastrointestinal *in vitro* favoreció la liberación de compuestos antioxidantes del bagazo fresco, mientras que en el bagazo deshidratado se produjo una disminución significativa de estos parámetros.

La fermentación colónica *in vitro* de los bagazos frescos y deshidratados utilizando inóculos de adultos sanos proporcionó un incremento de la actividad antioxidante y de los fenoles totales. Sin embargo, se requiere un seguimiento detallado de la biotransformación de los polifenoles para comprender este aumento y relacionarlo con los cambios observados en la microbiota intestinal. Asimismo, se necesita comprender el papel de otros nutrientes en la bioaccesibilidad y degradación de los polifenoles para proporcionar una visión completa de los tratamientos de deshidratación y el proceso digestivo.

La fermentación colónica del bagazo de almendra, fresco o deshidratado utilizando inóculos de adultos sanos promovió el crecimiento de Butyrivibrio, un género asociado a la degradación de fibras y producción de butirato, lo que podría tener implicaciones beneficiosas para la salud humana. Además, los métodos de deshidratación aplicados modificaron la estructura de la comunidad microbiana *in*

vitro; y específicamente el secado por aire caliente incrementó la presencia de géneros productores de ácidos grasos de cadena corta (SCFA).

El elevado contenido de proteínas en el bagazo trajo consigo la generación de ácidos grasos de cadena ramificada (BCFA) durante la fermentación colónica *in vitro* siendo los ácidos valérico e isovalérico los BCFA más abundantes. Sin embargo, la alta variabilidad entre individuos resalta la necesidad de ampliar el estudio con una muestra más representativa o con grupos con necesidades nutricionales específicas.

CAPÍTULO III

CAPÍTULO III: EL BAGAZO DE ALMENDRA COMO SUSTRATO DE UN ALIMENTO PROBIÓTICO. INFLUENCIA DEL PROCEDIMIENTO DE INCORPORACIÓN DE LAS CÉLULAS MICROBIANAS EN LA RESISTENCIA A LA DESHIDRATACIÓN Y DIGESTIÓN GASTROINTESTINAL *IN VITRO*.

ARTÍCULO 6

Duarte, S., Betoret, E., & Betoret, N. (2024). Strategies and mechanisms to increase the viability in non-dairy probiotic foods. Almond bagasse as a case study. **En preparación**.

RESUMEN CAPÍTULO III

La incorporación de microorganismos probióticos a alimentos no lácteos viene considerándose desde hace años como una estrategia para aumentar su valor funcional. Además, en los últimos años se ha incrementado el interés por llevar a cabo la fermentación de subproductos agroalimentarios con la finalidad de revalorizarlos y disminuir la generación de residuos. El proceso fermentativo permite aumentar la vida útil y desarrollar nuevos productos con propiedades nutricionales y funcionales mejoradas.

Los probióticos son microorganismos vivos que, cuando se administran en cantidades adecuadas, confieren un beneficio para la salud del huésped. Sin embargo, estos microorganismos deben alcanzar altos niveles de supervivencia tras el procesado y la digestión gastrointestinal para poder ejercer su efecto beneficioso. Por esta razón el objetivo de este capítulo es ofrecer una breve visión general sobre cómo el procesado de los alimentos y la digestión gastrointestinal *in vitro* afectan la supervivencia de células probióticos incluidas en los alimentos no lácteos; analizar el efecto de diferentes condiciones de estrés sobre la supervivencia y viabilidad de los microorganismos probióticos; y clasificar y detallar las estrategias que es posible aplicar en el desarrollo de alimentos probióticos no lácteos para aumentar el número de células viables; describir los mecanismos que promueven los cambios que sufren los microorganismos para sobrevivir y aumentar su viabilidad; presentar, como caso de estudio, los resultados obtenidos en la determinación del efecto del método de incorporación de *Lactobacillus salivarius* spp. (CECT4063) al bagazo de almendra fresco sobre la supervivencia al proceso de deshidratación y a la digestión gastrointestinal *in vitro*.

En el caso de estudio se utilizó bagazo de almendra como sustrato para el crecimiento del probiótico *Lactobacillus salivarius* spp. (CECT4063). Se evaluó el impacto de la matriz de incubación y las altas presiones de homogeneización (HPH) en su crecimiento, hidrofobicidad y resistencia a procesos como el secado por aire caliente y la digestión i*n vitro*. Los resultados indican que el bagazo de almendra conserva nutrientes y es adecuado como sustrato para el crecimiento de las células de *Lactobacillus*. El probiótico creció en todas las condiciones estudiadas y sobrevivió a la digestión *in vitro,* mostrando mejores resultados cuando fue encapsulado por HPH, lo que mejoró su resistencia a condiciones adversas como el secado por aire caliente y la digestión gastrointestinal *in vitro*.

ARTÍCULO 6: Strategies and mechanisms to increase the viability in non-dairy probiotic foods. Almond bagasse as a case study.

STRATEGIES AND MECHANISMS TO INCREASE THE VIABILITY IN NON-DAIRY PROBIOTIC FOODS. ALMOND BAGASSE AS A CASE STUDY.

The FAO/WHO definition of probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" has been widely adopted and has proven valuable to researchers, regulators and consumers. The panel of experts convened by the international scientific association for probiotics and prebiotics further considered two common general core benefits associated with probiotics: supporting a healthy digestive tract and a healthy immune system (Hill et al., 2014). Strains such as *Bifidobacterium* (*adolescentis, animalis, bifidum, breve and longum*) and *Lactobacillus* (*acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus* and *salivarius*) represent a core group of well-studied species likely to impart these general benefits (Hill et al., 2014). Such benefits are only obtained if the probiotic foods are consumed regularly and the food matrix contains a minimum amount of viable probiotic microorganisms. A level of 9 log colony-forming units (CFU) per day when delivered in food has been generally accepted for which non-strain specific claims might be. However, when specific strains, a level of 6 log CFU/g in food is accepted to confer a health effect (Bautista-Gallego et al., 2019).

The incorporation of probiotic microorganisms have been traditionally done into dairy foods. However, the incorporation into non-dairy foods with different structures is not new. Efforts have been already made to the incorporation of probiotic into different food matrices. Bakery, juices, fruits and vegetables foods with probiotics microorganisms have been studied. In order to have beneficial effects, probiotics microorganisms should survive the food processing operations but also the passage through the gastrointestinal tract (Ricciardi et al., 2014).However, food

processing conditions, storage and gastrointestinal pH levels affect negatively the survival of probiotic microorganisms, and the desired levels of 9 log CFU/g or mL are hardly reached.

Different strategies can be followed to reach high survival levels of microorganisms. The mechanisms by which probiotic microorganism are capable to survive and adapt to different stresses and harmful conditions are trying to be elucidated. The aim of this review is: (1) to give a brief overview on how food processing and gastrointestinal digestion affect the levels of probiotic microorganism included in non-dairy foods. (2) Analyze the effect of different stress conditions on the survival and viability of probiotic microorganisms. (3) Classify and detail the strategies that are being studied in the development of non-dairy probiotic foods to increase the viable cells number by their adaptation. (4) Describe the mechanisms that promote changes suffered by the microorganisms in order to survive and increase their viability. (5) To present, as a case study, the levels of probiotic microorganisms reached by different ways of incorporation into almond bagasse.

1. Effect of the processing and digestion on the survival of the probiotic microorganisms

In order to achieve and maintain the required quantity to have health effects, probiotic microbial cells need to survive the different stresses encountered during food manufacture as well as the harmful environments found throughout the gastrointestinal tract. Three different stages could be differentiated in the development of a probiotic food. The **first**, refers to the preparation, inoculation and growth of the probiotic microorganisms (that can be done into the food matrix or not). The **second**, refers to the processing operations (pretreatments, manufacturing, preservation and distribution) of food products from suitable raw

materials. The **third**, refers to the food consumption and its passage throughout the gastrointestinal digestion and the subsequent colonic fermentation. In all of three stages specific stresses determine the viability of probiotic microorganisms (Fiocco et al., 2020). The main stress factors that can have a major negative impact on probiotic viability.

***Stage 1. Preparation*.** Probiotic microorganisms can be cultivated in industrial media, later isolated and incorporated into the food. In this case, the optimum media growth ensures high yields of microorganisms managed to minimize the accumulation of inhibitory metabolic by-products that can difficult their growth. Major stresses may arise after the transference of microorganisms into food when suddenly exposed to an adverse environment and/or when stabilization or manufacture operations are needed.

On the other hand, when probiotic ferment the food matrix and transform it into de final food product, some stresses may be provided by the fermentation conditions: composition of the matrix that will be modified gradually, temperature, pH, salt concentration and oxygen presence. Native microorganisms are usually better adapted to fermentation processes, especially when fermentation is spontaneous. In some cases, the addition of starter cultures may be necessary to produce a probiotic food with a specific microbial population, or to ensure that the final product has specific physical, chemical and organoleptic properties. Interactions between native microbiota and probiotic starters can be critical in their viability. Metabolite competition, antagonistic or synergistic relations between microbial communities have been described. They have been mainly linked to the secretion of inhibitory substances as bacteriocins. In addition, the changes that raw material

undergoes during fermentation, with the depletion of available nutrients also limit their viability.

***Stage 2. Processing*:** Once the microorganisms are "grown" in the food with the highest/optimal quantity, the processing operations associated to the manufacture, distribution and storage can affect their viability too. The main stresses suffered by the microorganisms are thermal, osmotic and oxidative. The effect of the processing is highly strain dependent. Mainly, the changes in the cell membrane and damages to DNA and proteins due to the external stresses are those that determine cell viability. High temperatures above 50 °C, in drying, baking or cooking processes have effects on the microbial activity and growth of microorganisms by altering the fatty acids located in the bacterial membrane that are susceptible to heat damage. Because of the high temperatures, there is a protein denaturation due to the alteration of non-covalent interactions, which produces a subsequent aggregation of proteins and damage to ribosomes and RNA. On the other hand, exposures to low temperatures in freezing and storage, decreases microorganism's metabolism, inhibiting their growth (Champomier-Vergès et al., 2010; Corcoran et al., 2008). Loss of cell turgor, changes in solute concentration and changes in cell volume can result from osmotic stress during drying, fermentation, salting or concentration (Bisson et al., 2023). However, differences have been found between the effect of salt and sugar as well as in the ability of microorganisms to overcome the stress generated (Bisson et al., 2021). Exposure to oxygen during hot air drying, spray drying or mixing can form reactive oxygen species (ROS) which can cause damage by reacting with proteins, lipids and DNA (Corcoran et al., 2008; Li et al., 2010). Furthermore, in dried cells, oxidative stress can occur due to the oxidation of cell components, such as membrane lipid oxidation (Teixeira et al., 1996).

It has been possible to find in the literature studies about the incorporation of probiotic microorganisms into different non-dairy food matrices. *Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacterium animals* and *Saccharomyces boulardii* were the four species most commonly studied. The levels of probiotic microorganisms achieved in fruit and vegetable beverages were reviewed by Lillo-Pérez et al., (2021)and Rasika et al., (2021). The fruit sources used as probiotic substrates were pomegranate, orange, banana, lemon, tomato, watermelon, apricot, cherry, passion fruit and pineapple. The nutritious value of the fruits together with the possibility to modify the composition of the beverages easily ensured an adequate level of microorganisms after different storage time. Remarkable were the values of *Lactobacillus plantarum* found in pomegranate after 28 days of storage (10 log CFU/mL) (Mantzourani et al., 2018) and in pineapple juice (10 log CFU/mL)(Nguyen et al., 2019). In most of cases, levels of 6 log CFU/mL were achieved. However, the modification of media with ingredients that favor the growth of microorganisms, and the fermentation conditions usually gave undesirable sensorial characteristics in terms of flavor and texture. Some of the vegetable sources studied were moringa, carrot and artichoke. Other plant-based beverages studied were grain based on oat and rice, legume based on chickpea or soy, and nut based on almond, peanut or hazelnut. Levels of probiotic microorganisms between 6 - 8 CFU/mL were achieved in most of cases. The incorporation of probiotic in fruit and vegetable matrices have been studied too. In these cases, a liquid medium with high levels of microorganisms it is necessary to introduce the probiotics into fruit pieces by infusion, impregnation or osmotic dehydration. *Lactobacillus salivarius* spp. reached 6 log CFU/g in dried apples (Betoret et al., 2019). Similar values, between 5.5 and 6.5 log CFU/m of a lactobacillus mixed inoculum were found in guava and acerola fermented by products (de Oliveira et al., 2020). Mani-López et al., (2023)

reviewed in 2023 the studies about incorporation of probiotic microorganism into cereal based baked foods. The high temperatures achieved during baking made challenging the presence of viable probiotic microorganisms. *Lactobacillus plantarum* cells were reduced to 6.7 log CFU/g in cupcakes (Dong et al., 2020) and to 4-5 log CFU/g in pieces of bread with skim milk (Zhang et al., 2018) after 200 °C baking for 8-10 min. However, *Lactobacillus acidophilus* LA-5 did not exceed 3 log CFU/g in pieces of bread with skim milk and butter (Hadidi et al., 2021) and levels of 2 log CFU/g of *Bifidobacterium animalis* were reached in a loaf of white bread after 180 °C (Penhasi et al., 2021).

***Stage 3. Digestion*.** To have a beneficial effect, probiotic bacteria must be able to withstand gastric conditions such as low pH and varying concentrations of bile acids and enzymes, colonize the gastrointestinal tract adhering to the epithelial cells by adhesions on the surface of the bacteria. Exposure to acidic environments generally produces damage to the cell membrane, DNA and proteins (Amund, 2016). Proteins, polysaccharides and cell wall-associated components are part of the adhesion process. Resistance to acidic conditions is crucial in the adhesion of microorganisms. Ultimately, microorganisms could exert their health effects by producing antimicrobial compounds, competing for adhesion sites and nutrients with other species, and stabilizing the intestinal microflora. Naissinger da Silva et al., (2021) evaluated the probiotic viability of eleven commercial microorganisms, in the form of suspension or pills, after simulated gastrointestinal digestion and determined that all samples showed a reduction in the concentration of probiotic microorganisms and only six showed a concentration above 6 log CFU/g in the small intestine. (Islam et al., 2022) compared the effect of the simulated gastrointestinal digestion on free cells of *Lactobacillus acidophilus* and those included in chocolate matrix. The levels of

probiotic microorganisms after digestion were found to be 4 log CFU/g in those included in chocolate matrix but no viable cells where found in the suspended ones.

2. Strategies to increase the survival of probiotic microorganisms: protection and adaptation.

As presented above, in different food matrices, high levels of probiotic microorganisms, mostly in quantities of 8 log CFU/mL, have been successfully incorporated and maintained, especially in juices and beverages. However, after gastrointestinal digestion, even if in the best of cases survival levels of 80 % are achieved, the amounts of 6 log CFU/mL or g are hardly ever exceeded and amounts of 8 log CFU/mL or g are almost never reached. To increase the survival of probiotic microorganisms, new strategies beyond direct incorporation have been explored. Two lines of research can be highlighted. The first, involves the introduction of the microorganisms into structures than **protect** them from unfavorable conditions. The second, is based on the exposure of the microorganisms to certain conditions or external factors in such a way as to promote changes to favor their **adaptation**.

To **protect** the microorganisms from external unfavorable conditions by their incorporation into structures, the following strategies have been followed:

- Microencapsulation.

Microencapsulation can be defined as the process of coating tiny droplets of active ingredients with a micron size capsule. The capsule can protect the active ingredients against external stresses such as heat or acidity. Microencapsulation can also protect and increase the viability of probiotic microorganisms in food products and through the gastrointestinal passage. It represents a huge field of research in

which many advances have been achieved. Two reviews describe very well the progress that has been made in the last years. Sbehat et al. (2022) summarized the application of probiotic microencapsulation in different food matrices focusing on the protection against harmful conditions. Authors also highlighted the microencapsulation of probiotics with other functional ingredients as polyphenols, prebiotics or omega-3 fatty acids. (Vivek et al., 2023) reviewed the most used microencapsulation techniques, carriers and ongoing trends to develop non-dairy based probiotic fruit juice powder. There are not reviews about the probiotic's microencapsulation and the enhanced survival after *in vitro* gastrointestinal digestion. It was difficult to find studies in which the levels of probiotic microorganisms after simulated gastrointestinal conditions were bigger than 6 log CFU/mL. Microencapsulation in alginate improved the survival of *L. acidophilus* contained in soy and rice drinks to storage, stress conditions and *in vitro* gastrointestinal digestion at levels of 6 log CFU/mL (Angélica Andrade Lopes et al., 2020). In other study, encapsulation in casein and gum arabic improved the survival of *L. plantarum* under low pH, high temperature, alkaline conditions and storage in levels of 7-8 log CFU/mL. Although the survival of encapsulated cells after *in vitro* gastrointestinal digestion was bigger than free cells, the highest levels reached of microorganism were 4-5 log CFU/mL (Zong et al., 2023). Published research on specific probiotic strains confirmed the effectiveness of microencapsulation in increasing microbial resistance to gastrointestinal stress. The bigger drawback of microencapsulation in the incorporation of probiotic microorganisms into non-dairy foods are the sensory changes in the taste and texture of the food product.

To promote the **adaptation** of microorganisms by their exposure to controlled conditions, the following strategies have been followed:

- Strain selection and controlled fermentation towards directed evolution.

Directed evolution is a process based on Darwin's theory of natural selection. It involves adaptation to a highly stressful environment through spontaneous DNA mutations. Organisms that have acquired advantageous mutations will thrive, reproduce, and become dominant under the specific stress. The first step in directed evolution is the generation of a library of genetic variants (Packer & Liu, 2015). The ones that come closest to the desired value are then selected for use in breeding. Random mutagenesis or recombination is then used to generate a new generation of genotypic variants from this selected pool (Packer & Liu, 2015). Typically, as many rounds of this two-step process as necessary are performed iteratively. Biotechnological techniques have been developed and improved in recent years. These techniques can identify the genetic codes of the mutation acquired and can active, delete or modify the responsible genes increasing the throughput of the process and reducing human intervention (Esvelt et al., 2011; Wang et al., 2009), but the basic evolutionary process remains the same. However, genetic modification of organisms is not well accepted by consumers and is not allowed in many countries. Directed evolution has been applied to produce industrially desirable phenotypes such as increased resistance to stresses, including acid resistance (Overbeck et al., 2017)or ethanol tolerance (Betteridge et al., 2018). Directed evolution has been also applied to increase the production of certain compounds of interest such as ferulic acid (Liu et al., 2021) and flavonoids (Pandey et al., 2016) among others. Directed evolution has been also used to obtain food products with specific sensory characteristics, particularly in the production of wine (Guindal et al., 2023; Walker et al., 2022) and beer (Gallone et al., 2016).

- Controlled stress induction and subsequent incorporation.

Probiotics have been shown to increase their stress tolerance and viability when are exposed to a gradually increased sublethal conditions. Moreover, many studies showed that adapted *Lactobacillus* cells can develop cross-tolerances. For example, cells adapted to mild stress conditions have been demonstrated to increase their tolerance to the same or different exposed stresses such as high temperatures, acidity or oxygen presence (Ma et al., 2021; Ruiz et al., 2012; Settachaimongkon et al., 2015; Yang et al., 2021). Thus, making probiotics more resistant to challenging conditions such as food matrix, processing, storage, and gastrointestinal transit could be achieved by applying the appropriate exposure to adaptation condition. Different common probiotic *Lactobacillus* strain (*L. casei, L. acidophilus and L. plantarum*) have been subjected to challenging conditions such as acid pH (4-6.5), presence of salt (1-7 %) and sucrose (0.1-0.7 M) and their growth response have been evaluated (Bisson et al., 2023). In other cases, the *lactobacillus* strains has been isolated from typical fermented foods, such as *Lactobacillus kefiranofaciens* M1 isolated from Taiwanese kefir grains, and submitted to the effects of heat, cold, acid and bile salt conditions to improve its stress tolerance (Chen et al., 2017). The increased viability of adapted cells has been shown in some dairy foods such as yogurt or skimmed milk (Desmond et al., 2001; Settachaimongkon et al., 2015), but has not been proved in non-dairy ones yet. The strain and the growth limits for each condition tested have been strictly related to the specific microorganisms stress response (Ferrando et al., 2015).

When probiotic cells are exposed to sublethal stressful conditions, morphological changes can be observed. Changes in the morphological aspect of cells, such as elongated shapes or increases in cellular size have been identified under starvation or osmotic stress in *L. acidophilus* and *L. casei* (Piuri et al., 2005; Senz et al., 2015).

Superficial modifications cells of *L. salivarius* resulted in a hydrophobicity increase after the application of sublethal homogenization pressures or the inclusion of trehalose in mandarin juice (Betoret et al., 2017). Changes in the kinetics growth of the probiotic microorganisms has been observed after their submission to stressful conditions (Bisson et al., 2021). A correlation between cell morphology and strain stability during relevant processing steps such as extrusion, lyophilisation, freezing, dehydration and storage have been highlighted (Fonseca et al., 2000; Senz et al., 2015) Cell response to suboptimal growth conditions has been proved to be strain-dependent and can vary with the applied stress. Therefore, screening for the appropriate parameter to measure and determining the limiting values for each strain is mandatory (Gaucher et al., 2019).

To identify and understand the adaptation elements used by probiotic cells as well as to evaluate their subsequent metabolite production the analysis of gene or protein expression under stressful conditions can be a very useful tool. (Jung & Lee, 2020) analyzed the gene expression of *L. plantarum* under acidic conditions. Differentially expressed genes showed that transport function was affected by acidic conditions and determined that acidity during fermentation controlled the intracellular leucine transport. Proteomic analysis conducted in *L. kefiranofaciens* M1 subjected to different sublethal stresses revealed that 27 proteins were differently expressed in cells adapted or not (Chen et al., 2017).

3. Almond bagasse as a case study

Almond bagasse is a by-product generated in the production of the vegetable almond drink and which is currently used mainly for animal feed. However, this by-product retains a considerable amount of nutrients that make it a raw material of interest for revaluation. Fresh almond bagasse retained a considerable amount of

macronutrients after extracting almond beverage (Table 3.12). A reduction of 55% in fat, 56% in protein and 20% in fibre was observed when comparing the macronutrients values of whole almonds from the literature (de Oliveira Gonçalves et al., 2020; Yada et al., 2011, 2013)with those of bagasse. The high water activity and moisture values indicated that fresh almond bagasse was highly perishable. The low content of soluble solids was noteworthy, since the almond beverage was extracted with water. The pH was slightly above the pH range at which the *Lactobacillus salivarius* spp. is thought to be able to growth, from 2 to 7.4 (Rondón et al., 2008) Nevertheless, it should be noted that the microorganism can adapt easily to basic pH than to acidic one.

Table 3.12. Physicochemical properties and macronutrients content in fresh almond bagasse. Mean ± standard deviation of three repetitions.

a_w	X_w (g_{water}/g)	X_{ss} ($g_{soluble\ solids}/g$)	pH	Fat (g_{fat}/100 g)	Protein ($g_{protein}$/100 g)	Fiber Van Soest (g_{fibre}/100 g)
0.996 ± 0.001	0.818 ± 0.002	0.002 ± 0	7.62 ± 0.04	25.0 ± 0.2	9.9 ± 1.0	5.8 ± 0.5

In order to evaluate the feasibility of almond bagasse as a growth medium for *L. salivarius* spp. CECT 4063 three trials were performed under different conditions.

- In **TRIAL I**, the Man, Rogosa and Sharpe (MRS Broth) medium was inoculated (Scharlab, Barcelona, Spain) with *L. salivarius* spp. and incubated for 24 h at 37 °C. Then the almond bagasse was inoculated with ratio 1:10 (v/w) and incubated for 24 h at 37 °C.
- In **TRIAL II**, 2 L of MRS broth were inoculated and incubated for 24 h at 37 °C. After 24 h, the entire volume was centrifuged for 15 min, at 8000 rpm and 10 °C (Beckman Coulter AvantiTM J-25, California, United States). Subsequently, the supernatant was removed and the cells of the microorganism were suspended in 100 mL of water. The proportions

between the volume of broth, of resuspension water and mass of bagasse were calculated to get into the bagasse, before refrigeration, the same concentration of microbial cells as in TRIAL I.

- In **TRIAL III**, the procedure followed was the same as in TRIAL II with the difference than the cells suspended in 100 mL of water, were submitted to the encapsulation process by homogenization process following the methodology described in Betoret et al. (2019).

Hot air drying (HAD) of almond bagasse with probiotic microorganisms was carried out in an air dryer CLW 750 (Heratec, Wodzislaw, Polska) with air at 10 m/s at 50 °C until samples were reaching a water activity ≤ 0.3.

3.1. Effect of environmental conditions on the growth of the microorganism. Impact of drying and influence of encapsulation.

In the study of the viability of the probiotic, the ability of the microorganism to use almond bagasse as a growth medium was evaluated. As mentioned above, three different inoculation procedures were evaluated. Table 3.13 shows the growth values of the probiotic in the bagasse in the three evaluated conditions, and the variation caused by the drying operation with respect to FB1 and FB2.

Table 3.13. Viability of the microorganism (log CFU/g) in the different stages of the process. Mean ± standard deviation of three repetitions. Different superscripts letters mean statistically significant differences ($p \leq 0.05$).

	MRS	FB_1	FB_2	DB	$\left(\frac{DB\text{-}FB_1}{FB_1}\right)$	$\left(\frac{DB\text{-}FB_2}{FB_2}\right)$
TRIAL I	9.1 ± 0.3^{e}	8.9 ± 0.2^{d}	8.7 ± 0.3^{d}	7.8 ± 0.3^{ab}	-0.12	-0.10
TRIAL II	9.2 ± 0.2^{e}	8.2 ± 0.2^{c}	82 ± 0.2^{c}	7.6 ± 0.3^{ab}	-0.07	-0.08
TRIAL III	9.1 ± 0.3^{e}	8.0 ± 0.6^{b}	7.8 ± 0.3^{ab}	7.7 ± 0.5^{a}	-0.03	-0.01

TRIAL I: experimental procedure with fermentation
TRIAL II: experimental procedure with cell separation and incorporation
TRIAL III: experimental procedure with cell separation, HPH encapsulation and incorporation
FB: Fresh bagasse; **DB:** Dry bagasse.
$_1$: Before refrigeration; $_2$: 24 h refrigeration

Analysing the data in Table 3.13 and Figure 3.19, it can be seen that in the three cases the starting point is a similar concentration in the MRS culture medium. However, when the raw material FB1 is inoculated, a decrease in concentration is observed, being more noticeable in the TRIAL III and minimum in the TRIAL I. This fact shows the suitability of bagasse as a medium for bacterial growth. Although the pH is not optimal for the growth of the microorganism, the water content and the richness in nutrients make fermentation possible, and it was achieved after 24 h of incubation. The higher decrease in the other tests could be attributed to the experimental procedure. FB2 shows the content of microorganisms after cooling at 4 °C for 24 h of bagasse FB1. At this stage, no significant differences were observed for any of the tested conditions compared to the previous stage. Temperature is a critical parameter for the growth of microorganisms; refrigeration processes at temperatures of 4 °C generally cause the microorganism to enter a phase of dormancy with an absence of replication, stabilizing its concentration (Bernal Castro et al., 2017).

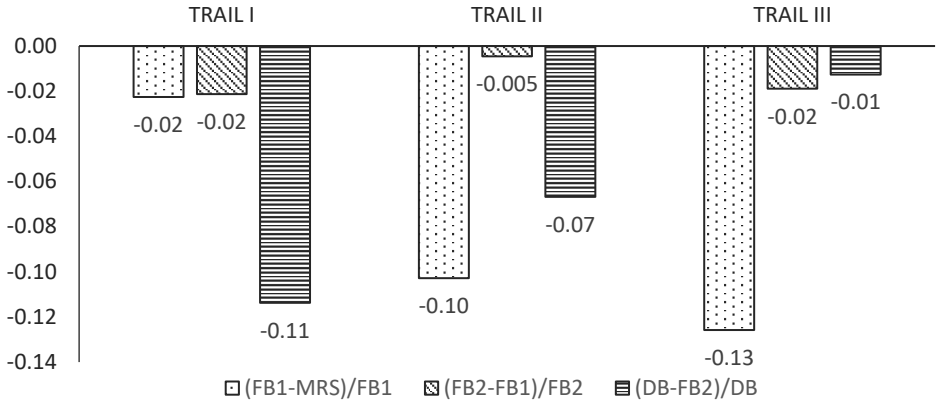

Figure 3.19. Loss of viability of microorganism after each stage of the process. BF1 relative to MRS, FB2 relative to FB1 and BS relative to FB2.

Finally, the content of microorganisms in DB samples was conditioned by the effect of drying on cell viability. Recent studies indicate that hot air drying processes around 40 - 60 °C negatively affect the viability of microorganisms with probiotic effect. Betoret et al. (2017) studied the viability of *L. salivarius* spp. in vacuum-impregnated apple snacks, reporting that the drying process did not contribute to a stabilisation of the microorganism, on the contrary, a temperature of 40 °C caused a decrease of 9 to 7 logarithmic units in the concentration of the probiotic.

The results indicated that after subjecting the samples to hot air drying at 50°C, the concentration of microorganisms decreased significantly when compared to the previous treatment (FB2), particularly in the TRIAL I and TRIAL III tests. However, in the TRIAL III, the loss of viability was minimal, potentially associated with the relatively low encapsulation efficiency achieved in the current trial (ranging between 55 and 57%). Nevertheless, this level of encapsulation was adequate to demonstrate that the encapsulation process effectively shields the cells from the elevated

temperatures applied during the drying operation. Similar findings were reported by Calabuig-Jiménez et al. (2019) where encapsulation proved instrumental in preserving the viability of the microorganism during the processing and storage of an apple snack inoculated with *Lactobacillus salivarius* spp. Additionally, encapsulation through high homogenization pressures (HPH) within the pressure range of 60 to 100 MPa has been shown to enhance the viability of probiotics in specific products (Siroli et al., 2020). In a general sense, while in FB1, fermentation played a pivotal role in maintaining the viability of the microorganism, in TRIAL III, it was the encapsulation process that significantly reduced the loss of viability.

3.2. Effect of growth conditions on resistance to gastro-intestinal digestion and the hydrophobicity of the probiotic.

Digestion is a complex process by which food ingested, after mechanical and enzymatic transformation decompose into chemically smaller and simpler substances, which the body can use for growth, cell maintenance and as fuel (Bas-Bellver et al., 2020). The disintegration takes place in the mouth and stomach, while the enzymatic digestion and subsequent absorption take place in the intestine thin and thick (Guerra et al., 2012).

The study of the digestive process is highly relevant since it allows obtaining useful information about the digestibility of food, the structural changes they undergo, the release of nutrients, the absorption of a food, the point of release of encapsulated materials, among others. Currently, there are both *in vivo* and *in vitro* models. *In vivo* models require a large amount of time, a high economic endowment and present ethical restrictions, due to the experimentation with living beings. The most commonly used *in vitro* models are laboratory-based assays, conducted in controlled environments outside of a living organism (Rivas Montoya, 2014). These

assays enable the flexible and reproducible replication of the physiological conditions that characterize human digestion (Guerra et al., 2012). Despite their limitations, the yield precise results within a short time frame and at a reduced cost (Coles et al., 2005). In this study, a gastrointestinal simulation was conducted on the product after inoculation (referred to as FB) and after undergoing a hot air drying treatment (referred to as DB) in the context of the three proposed trials. The viability of the microorganism was determined at the commencement and completion of the gastric phases (G1 and G2) as well as the intestinal phases (I1 and I2). The objective was to analyse variations in the microorganism's viability concerning inoculation conditions, the heat treatment applied, and the specific pH and enzymes employed in each phase of gastrointestinal digestion.

The FB sample displayed a higher solubility, readily diluting upon incorporation of the simulated fluids, making sampling easy. On the other hand, DB exhibited a denser character. Even after incorporating the simulated fluids, it did not fully dissolve, resulting in a pasty consistency that posed challenges for sampling and contributed to sample inhomogeneity. The results obtained from the *in vitro* digestion, as depicted in Table 3.14, indicated that the microorganism can withstand the gastrointestinal conditions experienced in all the studied scenarios. However, the final microorganism concentrations vary depending on the inoculation conditions in each of the trials.

Table 3.14. Viability of the microorganism throughout the gastrointestinal digestion process *in vitro* (log CFU/g). Mean ± standard deviation of three repetitions. Different superscripts letters mean statistically significant differences ($p \leq 0.05$).

		Initial	G_1	G_2	I_1	I_2
TRIAL I	**FB**	8.9 ± 0.2^{d}	8.0 ± 0.1^{bc}	8.1 ± 0.3^{bc}	7.7 ± 0.7^{b}	10.1 ± 0.2^{d}
	DB	7.8 ± 0.3^{ab}	8.2 ± 0.6^{c}	8.4 ± 0.2^{cd}	8.0 ± 0.5^{ab}	8.2 ± 1.0^{c}
TRIAL II	**FB**	8.2 ± 0.2^{c}	8.6 ± 0.5^{d}	8.6 ± 0.4^{d}	8.1 ± 0.7^{ab}	10.7 ± 0.2^{b}
	DB	7.7 ± 0.5^{a}	7.5 ± 0.4^{a}	7.6 ± 0.1^{a}	8.0 ± 0.2^{ab}	8.2 ± 0.3^{a}
TRIAL III	**FB**	8.0 ± 0.6^{bc}	$7.9 \pm 0.,5^{b}$	8.1 ± 0.5^{bc}	8.1 ± 0.6^{ab}	10.7 ± 0.1^{e}
	DB	7.7 ± 0.5^{a}	7.5 ± 0.2^{a}	7.8 ± 0.6^{ab}	8.9 ± 0.5^{a}	9.0 ± 0.4^{c}

FB: fresh bagasse; **DB:** dry bagasse.
G: gastric stage; **I:** intestinal stage.
$_1$: start of stage; **$_2$:** end of the stage.

In the initial results, there was a noticeable deviation one logarithmic unit between FB and DB. This discrepancy could be attributed to the heat treatment applied to the sample. As previously mentioned, the heat treatment adversely influences the viability of microorganisms, leading to a decrease in their concentration. In the early gastric stage (G1), a minor reduction in microorganism concentration was observed. The reduction was attributed to the decrease in pH, which shifted from 6.5 to 3 when HCl was introduced to the media. Notably, the TRIAL II-FB assay was the sole exception to this trend. These findings align with previous reports by Mahmoudi et al. (2016) and Escobar-Ramírez et al. (2020) in their viability studies of other *Lactobacillus* species. In the concluding gastric phase (G2), the concentration of the microorganism experienced an increase, with the notable exception of TRIAL III-FB, where the concentration remained constant. By the end of the gastric phase, it was evident that the microorganism successfully adapted to the pH change. This adaptation was attributed to the microorganism's ability to thrive within the optimal growth range of *L. salivarius* spp. In the early intestinal phase (I1) and early gastric phase (G1), the transition from pH 3 to 7 resulted in a reduction of the concentration of the microorganism in almost all samples. However, the

concentration remained stable in the TRIAL III-FB sample. An increase was observed in the TRIAL II-DB and TRIAL III-DB samples. Therefore, it can be considered that the microorganism showed an increased adaptability to low pH conditions. In the final intestinal phase (I2), a distinct trend emerged. TRIAL II exhibited a reduction in its concentration, more pronounced in DB than FB. In contrast, both TRIAL I and TRIAL III tests displayed an increase in the concentration of the microorganism. TRIAL III demonstrated a higher growth rate compared to TRIAL I. This disparity could be attributed to the HPH encapsulation process, which played a pivotal role in shielding the probiotic from adverse environmental conditions, particularly the pH variations encountered. Scientific studies, such as the one conducted by Calabuig-Jiménez et al. (2019), have corroborated the efficacy of this technique in bolstering the viability of microbial cells. The evolution of relative viability (defined as the content of microorganisms after each respective digestion stage divided by the content of microorganisms before the start of digestion) can be observed in Figure 3.20. It was evident that, at the final of the digestive process, both the TRIAL III tests in both FB and DB yield the highest concentrations of microorganisms, followed by the TRIAL I test.

These results can be explained by the protective impact of encapsulation on microbial cells within the TRIAL III, mitigating the harsh conditions of the gastrointestinal tract. In the case of the TRIAL I, the microorganisms' adaptation to the solid medium of bagasse, along with their initial growth in this substrate, may have bolstered their resilience against the unfavourable conditions associated with the digestive process. Conversely, in the TRIAL II test, where neither of the aforementioned circumstances is at play, the resistance was diminished.

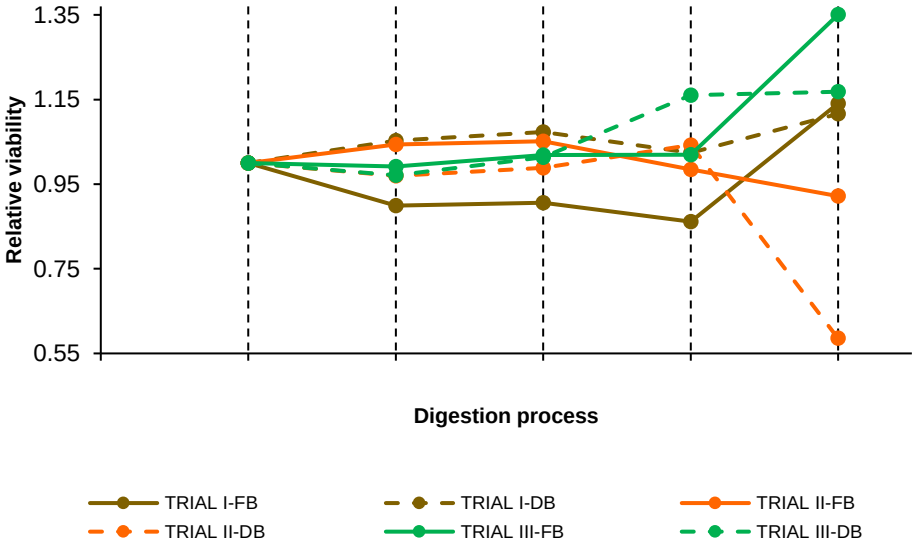

Figure 3.20. Relative evolution of *Lactobacillus salivarius* spp. content throughout the gastrointestinal digestion process in fresh and dry samples from each of the trials.

In probiotic microorganisms, cell hydrophobicity is mainly related to the ability of the microorganism to interact with the intestinal mucosa, thus being able to exert its effect (Kos et al., 2003; Savedboworn et al., 2020). It is necessary for the microorganism to have a hydrophobicity higher than 40% to be considered as a probiotic (Del Re et al., 2000). Studies on the genus Lactobacillus indicate a hydrophobicity between 32 - 70% (Sivamaruthi et al., 2020; Vijayalakshmi et al., 2020; Vinderola & Reinheimer, 2003). Specifically, in the study conducted by Betoret et al. (2017) on the hydrophobicity of the probiotic *Lactobacillus salivarius* spp. incubated in MRS broth, values of 66.9 % were obtained.

The hydrophobicity values of the microorganism incubated in almond bagasse that were higher than the 40% required to be called probiotic (Figure 3.21). Statistical analysis shows significant differences between values obtained. This variability

showed a greater hydrophobicity in the test TRIAL I and TRIAL III, compared to a lower value in TRAI II. Generally, variation in hydrophobicity is related to the change in bacterial wall structure when the microorganism is subjected to stress conditions such as pH variations or high temperatures (Remeta et al., 2002). TRIAL I and TRIAL III tests showed higher values. This could be associated with the fact that in the TRAI I test, the microorganism undergoes an adaptation process with consequent changes in its structure. The TRIAL III assay, by subjecting the microorganism to HPH stress, contributed to a better adaptation to hydrophobic conditions, in both cases conferring to the probiotic a higher hydrophobicity and consequently a better adhesion to the intestinal epithelium. Studies by Tabanelli et al. (2014) and Betoret et al. (2017) on probiotic bacteria showed that HPH treatment at 50 MPa can contribute to improve certain functional properties such as hydrophobicity, aggregation and resistance to biological stress, thus preserving the viability of the microorganism during storage and making this technology an option to consider for microencapsulating probiotics.

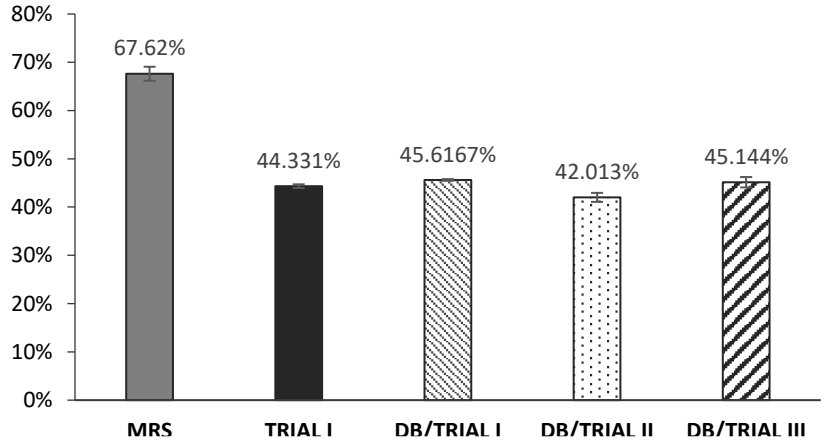

Figure 3.21. Hydrophobicity of *Lactobacillus salivarius* spp. under the different conditions tested. Values are the average result of four replicates. Mean ± standard deviation of three repetitions.

3.3. Conclusions

The nutrients of interest such as proteins, lipids and fibre preserved in bagasse made it a raw material of interest as a culture medium for probiotics. Among the three trials tested, the TRIAL III was the one with the best results, showing that encapsulation contributes positively to maintaining the viability of the microorganism under the adverse conditions of hot air drying. The results of the *in vitro* digestion showed that the microorganism was able to survive the variations experienced in the different phases of the digestion in the three tests, being again the TRIAL III test the one that provided a higher viability of the microorganism at the end of the digestive process.

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CONCLUSIONES CAPÍTULO III

En alimentos probióticos no lácteos difícilmente se alcanzan las cantidades óptimas de microorganismos (9 log CFU/mL o g) después de las operaciones de procesado, almacenamiento y digestión gastrointestinal *in vitro* y solo en algunas ocasiones se alcanzan las mínimas necesarias (6 log CFU/mL o g) para ejercer un efecto potencialmente beneficioso sobre la salud.

En la revisión bibliográfica se identificaron las etapas necesarias para el desarrollo de un alimento probiótico, así como los factores de estrés asociados a cada una de ellas. Además, se han clasificado las posibles estrategias que se están llevando a cabo en los últimos años dirigidas a aumentar la viabilidad y supervivencia de los microorganismos probióticos. Estas estrategias se han dividido en dos grandes grupos. Por un lado, las estrategias basadas en la protección de las células microbianas por su inclusión en estructuras protectoras. Por otro lado, se han identificado y descrito las estrategias basadas en la exposición de los microorganismos probióticos a condiciones desfavorables, pero no letales, para favorecer la aparición de cambios en los mismos que promuevan su adaptación.

El bagazo resultante de la obtención de la bebida de almendra ha mostrado ser un sustrato adecuado para el crecimiento de la cepa probiótica *L. salivarius* spp. Las diferentes formas de incorporación al sustrato influyeron en los niveles de microorganismos alcanzados después de la operación de secado por aire caliente. Los resultados de la digestión gastrointestinal *in vitro* revelaron la viabilidad del microorganismo después de las distintas fases de digestión. En ambos casos, los resultados evidenciaron que la encapsulación de *L. salivarius* spp. en un recubrimiento de alginato mediante la utilización de las presiones de homogeneización permitió obtener los niveles más elevados del mismo.

4. PERSPECTIVAS A FUTURO

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Los resultados obtenidos en la presente tesis doctoral han demostrado el potencial uso del residuo procedente de la obtención de la bebida vegetal de almendra como sustrato para la obtención de ingredientes funcionales de interés para la industria alimentaria. Esto debido a su composición, a sus propiedades fisicoquímicas y tecnológicas y a su idoneidad para ser transformado en un producto en polvo estable, con adecuadas propiedades y con un potencial efecto favorable sobre la microbiota intestinal. No obstante, sería conveniente realizar estudios relacionados con la incorporación de antiapelmazantes que facilitaran la operación de triturado y la obtención de polvos de diferente granulometría.

Los polvos de bagazo de almendra presentaron un buen perfil nutricional, destacando su alto contenido en fibra, proteína y antioxidantes, además de un alto contenido de grasa. Sería recomendable realizar un análisis del perfil proteico y de ácidos grasos con la finalidad de determinar con mayor precisión las aplicaciones más recomendable y establecer su evolución durante el almacenamiento.

Los ensayos de digestión gastrointestinal *in vitro* y de fermentación colónica han evidenciado el potencial de los diferentes polvos como ingredientes funcionales para mejorar el valor nutricional en la formulación de alimentos. No obstante, sería importante llevar a cabo estudios de digestión *in vitro* que determinen la digestibilidad y la biodisponibilidad de nutrientes y compuestos bioactivos al integrar los polvos de bagazo de almendra en otras estructuras alimentarias, y que abarquen la fermentación colónica con individuos que presenten necesidades específicas. Estos estudios permitirían comprender y mejorar los posibles efectos beneficiosos que podrían tener en la salud.

PERSPECTIVAS A FUTURO

Por lo que respecta a las posibles aplicaciones de los polvos, se ha comprobado que resultan idóneos para su incorporación en productos de panadería como las galletas, dando lugar a un producto final con mejores propiedades nutricionales. No obstante, convendría realizar estudios sensoriales y tener en cuenta otro tipo de aplicaciones que permitieran diversificar su uso.

La fermentación del bagazo fresco podría ser una alternativa viable para su revalorización en forma de alimento probiótico con elevado valor funcional. Convendría realizar ensayos específicos que permitieran aumentar la resistencia de *Lactobacillus salivarius* spp. a condiciones desfavorables de temperatura, acidez y presión.

Finalmente, este tipo de aproximaciones puede contribuir de manera efectiva al desarrollo de alimentos más saludables y sostenibles. Sin embargo, es necesario realizar pruebas a mayor escala como paso previo a su producción a nivel industrial.