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TESIS DOCTORAL

MEJORA DE LA CALIDAD FUNCIONAL DE UN SNACK CON EFECTO PROBIÓTICO Y ANTIOXIDANTE MEDIANTE LA INCORPORACIÓN DE TREHALOSA Y LA APLICACIÓN DE ALTAS PRESIONES DE HOMOGENEIZACIÓN

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Valencia, 27 diciembre de 2022

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Las **Dras. Cristina Barrera Puigdollers y Noelia Betoret Valls**, Profesoras Titulares de Universidad, pertenecientes al Departamento de Tecnología de los Alimentos de la Universitat Politècnica de València y **directoras de la presente tesis doctoral**,

CONSIDERAN que la memoria titulada **"Mejora de la calidad funcional de un snack con efecto probiótico y antioxidante mediante la incorporación de trehalosa y la aplicación de altas presiones de homogeneización"** que presenta Dña. Cristina Gabriela Burca Busaga para aspirar al grado de Doctora por la Universitat Politècnica de València, y que ha sido realizada bajo su dirección en el Instituto Universitario de Ingeniería de Alimentos para el Desarrollo de la Universitat Politècnica de València, reúne las condiciones adecuadas para constituir su tesis doctoral, por lo que **AUTORIZAN** su presentación por parte de la interesada.

Valencia, diciembre de 2022

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Fdo. Noelia Betoret Valls

A mis padres Joan y Zamfira (Q.E.P.D)

por inculcarme el valor del esfuerzo

A mi marido Ramón con todo el cariño

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Estancia nacional de investigación en el Departamento de Biotecnología del Instituto de Agroquímica y Tecnología de los Alimentos (IATA-CSIC), desde el 28 de mayo de 2018 hasta el 8 de junio de 2018, bajo la supervisión de la Dra. María Carmen Collado Amores.

RESUMEN

Uno de los desafíos clave que se presenta en el siglo XXI es la necesidad de alimentar a una población cada vez mayor con recursos naturales cada vez más limitados. Adicionalmente, el Objetivo de Desarrollo Sostenible (ODS) 12 de la Agenda 2030 aboga por formas de producción y consumo más sostenibles. En este sentido, se favorece el aprovechamiento y desarrollo de alimentos de origen vegetal frente a los de origen animal. Por otra parte, hay estudios que demuestran que la ingesta de determinados microorganismos en cantidades adecuadas produce efectos beneficiosos para la salud de los consumidores y que la fermentación con estos microorganismos contribuye en la mejora de las propiedades nutricionales de diferentes matrices alimentarias.

Debido a esto, en los últimos años ha aumentado el interés de los científicos por desarrollar alimentos funcionales probióticos a partir de matrices vegetales, destinados a responder también a las necesidades nutricionales de los consumidores vegetarianos, con intolerancia a lactosa y con dietas restrictivas por el elevado nivel de colesterol. Entre las técnicas empleadas, la impregnación a vacío permite la incorporación de microorganismos probióticos en la matriz estructural de frutas y hortalizas sin alterar su integridad celular. Este hecho, sin embargo, se traduce en mejores recuentos tras el procesado y el almacenamiento, pero no es suficiente para garantizar la viabilidad de los microorganismos tras la estabilización mediante secado por aire caliente, lo que hace necesario recurrir a otras estrategias. Concretamente en esta tesis doctoral las técnicas que se aplican para aumentar la resistencia de los probióticos a las condiciones adversas de procesado de los alimentos son la adición de agentes protectores, como la trehalosa, y la aplicación de altas presiones de homogeneización (HPH).

Los resultados de las investigaciones se presentan por compendio de 4 artículos científicos organizados en dos capítulos. En el Capítulo I “Obtención de un líquido de impregnación a base de zumo de clementina con elevado contenido en unidades formadoras de colonias de *L. salivarius* CECT 4063 que sean resistentes al almacenamiento y a la digestión simulada *in vitro* se ha analizado el efecto que la concentración de trehalosa (0-20%, p/p) y la presión de homogeneización (0-150 MPa) ejercen sobre las propiedades antioxidantes y el crecimiento de la cepa de *L. salivarius* spp*. salivarius* CECT 4063 en zumo de clementina comercial, así como sobre la capacidad del microorganismo para inhibir al patógeno *Helicobacter pylori*, para adherirse a la mucosa intestinal y para resistir el proceso de digestión *in vitro*. También se ha evaluado cómo la propia fermentación con el lactobacilo afecta al contenido en compuestos con actividad antioxidante del zumo, en mayor o menor medida dependiendo de la adición o no de un 10% en peso de trehalosa al medio y/o de la homogeneización a 100 MPa del zumo fermentado y sin fermentar. Por último, se ha estudiado la evolución de las propiedades antioxidantes y microbianas de los zumos fermentados durante su almacenamiento en refrigeración, así como su habilidad para ser incorporados en la matriz estructural de láminas de manzana (var. *Granny Smith*) mediante la técnica de impregnación a vacío. A partir de los ensayos realizados se ha podido constatar que:

- La homogeneización a 100 MPa del zumo de clementina antes de la inoculación con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 mejoró notablemente el

crecimiento microbiano, pero aceleró la degradación de los compuestos antioxidantes.

- El empleo de zumo de clementina comercial como medio para el crecimiento de *Lactobacillus salivarius* spp*. salivarius* CECT 4063 no mejoró significativamente su habilidad para inhibir el crecimiento del patógeno *Helicobacter pylori*, pero sí su capacidad para adherirse a mucina, colágeno y albúmina de suero bovino.
- Una vez elaborado el líquido de impregnación, éste no debería almacenarse durante más de 15 días en refrigeración para mantener los recuentos por encima de 10^7 UFC/mL y conservar su condición de probiótico.

Por otra parte, en el Capítulo II "Obtención de un snack de manzana (var. *Granny Smith*) con elevado contenido en compuestos antioxidantes y en unidades formadoras de colonias de *L. salivarius* CECT 4063" mediante impregnación a vacío y posterior liofilización o secado con aire a 40 °C se ha analizado el efecto que la adición de un 10% (p/p) de trehalosa al zumo de clementina antes de su inoculación con el microorganismo y/o la homogeneización a 100 MPa del zumo de clementina fermentado ejercen sobre la viabilidad del lactobacilo y la estabilidad de los compuestos antioxidantes frente al procesado, el almacenamiento y la digestión simulada *in vitro.* A partir de los ensayos realizados se ha podido constatar que:

- Tanto la liofilización como el secado con aire a 40 °C durante 12 y 24 h ejercieron un impacto negativo sobre el contenido en *Lactobacillus salivarius* CECT 4063, pero mejoraron las propiedades antioxidantes de las muestras impregnadas.
- La población microbiana en los snacks de manzana disminuyó a los 30 días de almacenamiento a temperatura ambiente. De entre las muestras analizadas, las obtenidas mediante secado con aire a 40 °C hasta una actividad del agua de 0,35 fueron las que menor reducción experimentaron en la población microbiana, especialmente tras añadir trehalosa al líquido de impregnación.
- De los estudios de digestión *in vitro* se deduce que tanto la supervivencia de *Lactobacillus salivarius* CECT 4063 como la bioaccesibilidad de los antioxidantes se vieron afectadas por la matriz alimentaria de la que forman parte.
- La homogeneización a 100 MPa del zumo de clementina fermentado empleado como líquido de impregnación también aumentó la supervivencia del *Lactobacillus salivarius* CECT 4063 durante la digestión *in vitro* de las muestras de manzana liofilizadas, aunque los compuestos con actividad antioxidante alcanzaron una mayor concentración en las muestras deshidratadas que en las simplemente impregnadas.

En conclusión, se ha comprobado que la adición de trehalosa, la aplicación de HPH y la inclusión en una matriz alimentaria son estrategias adecuadas para la mejora de la calidad funcional de un snack de manzana impregnado con zumo de clementina fermentado con *Lactobacillus salivarius* spp*. salivarius* CECT 4063. Los alimentos probióticos con matriz vegetal se presentan como el desafío industrial más novedoso, para cubrir un nicho de mercado creciente y muy prometedor que claramente exige condiciones estrictas para sus productos.

ABSTRACT

One of the key challenges of the 21st century is the need to feed a growing population with increasingly limited natural resources. Additionally, Sustainable Development Goal (SDG) 12 of the 2030 Agenda advocates for more sustainable forms of production and consumption. In this sense, the use and development of plant-based foods is favoured over those of animal origin. On the other hand, there are studies showing that the intake of certain microorganisms in adequate amounts produces beneficial effects for the health of consumers and that fermentation with these microorganisms contributes to the improvement of the nutritional properties of different food matrices.

Due to this, in recent years the interest of scientists has increased in developing probiotic functional foods from plant matrices, aimed at also responding to the nutritional needs of vegetarian consumers, with lactose intolerance and with restrictive diets due to the high cholesterol level. Among the techniques used, vacuum impregnation allows the incorporation of probiotic microorganisms in the structural matrix of fruits and vegetables without altering their cellular integrity. This fact, however, translates into better counts after processing and storage, but it is not enough to guarantee the viability of the microorganisms after stabilization by hot air drying, which makes it necessary to resort to other strategies. Specifically, in this doctoral thesis, the techniques applied to increase the resistance of probiotics to adverse food processing conditions are the addition of protective agents, such as trehalose, and the application of high pressure homogenization (HPH).

The results of the investigations are presented by compendium of 4 scientific articles organized in two chapters. In Chapter I "Obtaining an impregnation liquid based on clementine juice with a high content of colony-forming units of *L. salivarius* spp. *salivarius* CECT 4063 that are resistant to storage and "*in vitro*" simulated digestion, the effect that the trehalose concentration (0-20%, w/w) and the homogenization pressure (0-150 MPa) exert on the antioxidant properties and the growth of the *L. salivarius* spp. *salivarius* CECT 4063 in commercial clementine juice, as well as on the capacity of the microorganism to inhibit the pathogen *Helicobacter pylori*, to adhere to the intestinal mucosa and to resist the *in vitro* digestion process. It has also been evaluated how the fermentation itself with this *Lactobacillus* strain, affects the content of compounds with antioxidant activity in the juice, to a greater or lesser extent depending on the addition or not of 10% by weight of trehalose to the medium and/or the homogenization to 100 MPa of fermented and unfermented juice. Finally, the evolution of the antioxidant and microbial properties of fermented juices during refrigerated storage has been studied, as well as their ability to be incorporated into the structural matrix of apple slices (var. *Granny Smith*) using the vacuum impregnation technique. From the tests carried out, it has been found that:

- Homogenization at 100 MPa of clementine juice before inoculation with *Lactobacillus salivarius* CECT 4063 markedly improved microbial growth but accelerated the degradation of antioxidant compounds.
- The use of commercial clementine juice as a medium for the growth of *Lactobacillus salivarius* CECT 4063 did not significantly improve its ability to inhibit the growth of the pathogen *Helicobacter pylori*, but its ability to adhere to mucin, collagen, and bovine serum albumin.

- Once the impregnation liquid has been prepared, it should not be stored for more than 15 days in the refrigerator to keep the counts above 10^7 CFU/mL and preserve its probiotic status.

On the other hand, in Chapter II "Obtaining an apple snack (var. *Granny Smith*) with a high content of antioxidant compounds and colony-forming units of *L. salivarius* CECT 4063" by vacuum impregnation and subsequent lyophilization or drying with air at 40 °C, the effect that the addition of 10% (p/p) trehalose to clementine juice before inoculation with the microorganism and/or homogenization at 100 MPa of the fermented clementine juice has been analysed on the viability of lactobacillus and the stability of antioxidant compounds against processing, storage and simulated digestion *in vitro*. From the tests carried out, it has been found that:

- Both lyophilization and air-drying at 40 °C for 12 and 24 h had a negative impact on the content of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 but improved the antioxidant properties of the impregnated samples.
- The microbial population in apple snacks decreased after 30 days of storage at room temperature. Among the samples analysed, those obtained by drying with air at 40 °C to a water activity of 0.35 were the ones that experienced the least reduction in the microbial population, especially after adding trehalose to the impregnation liquid.
- From the *in vitro* digestion studies, it can be deduced that both the survival of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 and the bioaccessibility of the antioxidants were affected by the food matrix of which they are a part.
- Homogenization at 100 MPa of the fermented clementine juice used as impregnation liquid also increased the survival of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 during *in vitro* digestion of freeze-dried apple samples, although compounds with antioxidant activity reached a higher concentration in dehydrated than in simply impregnated samples.

In conclusion, it has been verified that the addition of trehalose, the application of HPH and the inclusion in a food matrix are suitable strategies for improving the functional quality of an apple snack impregnated with clementine juice fermented with *Lactobacillus salivarius* spp. *salivarius* CECT 4063. Plant-based probiotic foods are presented as the newest industrial challenge, to cover a growing and very promising market niche that clearly demands strict conditions for its products.

RESUM

Un dels desafiaments clau que es presenta en el segle XXI és la necessitat d'alimentar a una població humana cada vegada major amb recursos naturals cada vegada més limitats. Addicionalment, l'Objectiu de Desenvolupament Sostenible (ODS) 12 de l'Agenda 2030 advoca per formes de producció i consum més sostenibles. En aquest sentit, s'afavoreix l'aprofitament i desenvolupament d'aliments d'origen vegetal enfront dels d'origen animal. D'altra banda, hi ha estudis que demostren que la ingesta de determinats microorganismes en quantitats adequades produeix efectes beneficiosos per a la salut dels consumidors i que la fermentació amb aquests microorganismes contribueix a la millora de les propietats nutricionals de diferents matrius alimentàries.

A causa d'això, en els últims anys ha augmentat l'interès dels científics per desenvolupar aliments funcionals probiòtics a partir de matrius vegetals, destinats a respondre també a les necessitats nutricionals dels consumidors vegetarians, amb intolerància a la lactosa i amb dietes restrictives per l'elevat nivell de colesterol. Entre les tècniques emprades, la impregnació a buit permet la incorporació de microorganismes probiòtics en la matriu estructural de fruites i hortalisses sense alterar la seua integritat cel·lular. Aquest fet, però, es tradueix en millors recomptes després del processament i l'emmagatzematge, però no n'hi ha prou per garantir la viabilitat dels microorganismes després de l'estabilització mitjançant assecat per aire calent, cosa que fa necessari recórrer a altres estratègies. Concretament en aquesta tesi doctoral les tècniques que s'apliquen per a augmentar la resistència dels probiòtics a les condicions adverses de processament són l'addició d'agents protectors, com la trehalosa, i l'aplicació d'altes pressions d'homogeneïtzació (HPH).

Els resultats de les investigacions es presenten per compendi de 4 articles científics organitzats en dos capítols. En el Capítol I "Obtenció d'un líquid d'impregnació a base de suc de clementina amb elevat contingut en unitats formadores de colònies de *L. salivarius* spp. *salivarius* CECT 4063 que siguin resistents a l'emmagatzematge i a la digestió simulada *in vitro*" s'ha analitzat l'efecte que la concentració de trehalosa (0-20%, p/p) i la pressió d'homogeneïtzació (0-150 MPa) exerceixen sobre les propietats antioxidants i el creixement del cep de *L. salivarius* spp. *salivarius* en suc de clementina comercial, així com sobre la capacitat del microorganisme per a inhibir al patogen *Helicobacter pylori*, per a adherir-se a la mucosa intestinal i per a resistir el procés de digestió *in vitro*. També s'ha avaluat com la pròpia fermentació amb el lactobacilo afecta al contingut en compostos amb activitat antioxidant del suc, en major o menor mesura depenent de l'addició o no d'un 10% en pes de trehalosa al medi i/o de l'homogeneïtzació a 100 MPa del suc fermentat i sense fermentar. Finalment, s'ha estudiat l'evolució de les propietats antioxidants i microbianes dels sucs fermentats durant el seu emmagatzematge en refrigeració, així com la seua habilitat per a ser incorporats en la matriu estructural de làmines de poma (var. *Granny Smith*) mitjançant la tècnica d'impregnació a buit. A partir dels assajos realitzats s'ha pogut constatar que:

- L'homogeneïtzació a 100 MPa del suc de clementina abans de la inoculació amb *Lactobacillus salivarius* CECT 4063 va millorar notablement el creixement microbià, però va accelerar la degradació dels compostos antioxidants.
- L'ús de suc de clementina comercial com a mitjà per al creixement de *Lactobacillus salivarius* CECT 4063 no va millorar significativament la seua

habilitat per a inhibir el creixement del patogen *Helicobacter pylori*, però sí la seua capacitat per a adherir-se a la mucina, el col·lagen i l'albúmina de sèrum boví.

- Una vegada elaborat el líquid d'impregnació, aquest no hauria d'emmagatzemar-se durant més de 15 dies en refrigeració per a mantindre els recomptes per damunt de 10^7 UFC/mL i conservar la seua condició de probiòtic.

D'altra banda, en el Capítol II "Obtenció d'un snack de poma (var. *Granny Smith*) amb elevat contingut en compostos antioxidants i en unitats formadores de colònies de *L. salivarius* CECT 4063" mitjançant impregnació a buit i posterior liofilització o assecat amb aire a 40 °C s'ha analitzat l'efecte que l'addició d'un 10% (p/p) de trehalosa al suc de clementina abans de la seua inoculació amb el microorganisme i/o l'homogeneïtzació a 100 MPa del suc de clementina fermentat exerceixen sobre la viabilitat del lactobacilo i l'estabilitat dels compostos antioxidants enfront del processament, l'emmagatzematge i la digestió simulada *in vitro*. A partir dels assajos realitzats s'ha pogut constatar que:

- Tant la liofilització com l'assecat amb aire a 40 °C durant 12 i 24 h van exercir un impacte negatiu sobre el contingut en *Lactobacillus salivarius* spp. *salivarius* CECT 4063, però van millorar les propietats antioxidants de les mostres impregnades.
- La població microbiana en els snacks de poma va disminuir als 30 dies d'emmagatzematge a temperatura ambient. D'entre les mostres analitzades, les obtingudes mitjançant assecat amb aire a 40 °C fins a una activitat de l'aigua de 0,35 van ser les que menor reducció van experimentar en la població microbiana, especialment després d'afegir trehalosa al líquid d'impregnació.
- Dels estudis de digestió *in vitro* es dedueix que tant la supervivència de *Lactobacillus salivarius* CECT 4063 com la bioaccesibilitat dels antioxidants es van veure afectades per la matriu alimentària de la qual formen part.
- L'homogeneïtzació a 100 MPa del suc de clementina fermentat empleat com a líquid d'impregnació també va augmentar la supervivència del *Lactobacillus salivarius* CECT 4063 durant la digestió *in vitro* de les mostres de poma liofilitzades, encara que els compostos amb activitat antioxidant van aconseguir una major concentració en les mostres deshidratades que en les simplement impregnades.

En conclusió, hem comprovat que l'addició de trehalosa, l'aplicació d'HPH i la inclusió en una matriu alimentària són estratègies adequades per a la millora de la qualitat funcional d'un snack de poma impregnat amb suc de clementina fermentat amb *Lactobacillus salivarius* spp. *salivarius* CECT 4063. Els aliments probiòtics amb matriu vegetal es presenten com el desafiament industrial més nou, per a cobrir un nínxol de mercat creixent i molt prometedor que clarament exigeix condicions estrictes per als seus productes.

ÍNDICE

I. INTRODUCCIÓN

I.1. Evolución histórica en los conceptos y el consumo de probióticos y prebióticos

I.1.1. Probióticos, prebióticos y sinbióticos

Los probióticos y los prebióticos han recibido una atención cada vez mayor en los últimos años en los ámbitos científico, sanitario y público. La publicidad en torno a la investigación del microbioma también ha ampliado la percepción pública y la comprensión de las funciones beneficiosas de los microorganismos en la salud humana. De acuerdo con estos avances, continúa el crecimiento de la industria de probióticos (estimado en un 7% anual) y prebióticos (pronosticado en un 12,7% anual) durante los próximos ocho años (Cunningham et al., 2021). Este hecho se ve también reflejado en la literatura científica, de manera que el número de publicaciones relacionadas con los probióticos creció desde 176 en el año 1965, hasta 1.476 en 2014, incluyendo 477 ensayos médicos aleatorizados (según PubMed). En febrero de 2019, el número de publicaciones que tenían como tema de investigación los probióticos fue de 20.315 (Georgieva et al., 2022).

La historia de los probióticos es tan antigua como la humanidad y está estrechamente relacionada con el uso de los alimentos fermentados (Gasbarrini et al., 2016). La fermentación, “fermentum” en latín, es el proceso más antiguo de conservación de los alimentos mediante el cual se potencia el sabor y aumenta su digestibilidad. Según los descubrimientos arqueológicos llevados a cabo en diferentes partes del mundo, la producción de alimentos fermentados comenzó en torno al año 10000 A.C. (Ilango y Antony, 2021), aunque el proceso de fermentación no empezó a ser investigado hasta el siglo XIX, cuando Louis Pasteur identificó los microorganismos responsables de estas reacciones (Acevedo-Díaz y García-Carmona, 2016).

A principios del siglo XX Elie Metchnikoff, científico ruso Nobel en Medicina por sus trabajos en el Instituto Pasteur de París, dio por primera vez una explicación científica de los efectos beneficiosos de las bacterias del ácido láctico (BAL) presentes en la leche fermentada y afirmó que “los microorganismos de los alimentos permiten modificar la flora de nuestro cuerpo y reemplazar los microbios dañinos por microbios útiles” (Sarao y Arora, 2017).

El término "probiótico" proviene de dos términos, el primero del latín “pro" que significa "a favor" y el segundo del término griego “βιοσ" que significa "vida” (“a favor de la vida”) y se utilizó por primera vez en 1954 para comparar los efectos nocivos de los antibióticos con los beneficiosos de determinadas bacterias (Georgieva et al., 2022). En 2001, la Organización para la Agricultura y la Alimentación y la Organización Mundial de la Salud (FAO/OMS, 2001), aprobaron las directrices en relación con la evaluación de los probióticos:

- Los microorganismos probióticos utilizados en los alimentos deberán ser capaces de sobrevivir al paso por el tracto digestivo y también proliferar en el intestino.
- Unida a la propiedad arriba mencionada, los probióticos deberían ser resistentes a los jugos gástricos y poder crecer en presencia de bilis, en las condiciones existentes en los intestinos, o ser consumidos en un alimento que actúa como

vehículo y les permita vivir al paso por el estómago y a la exposición de bilis (FAO/OMS, 2001).

Estos requisitos están avalados por la Asociación Científica Internacional de Probióticos y Prebióticos (ISAPP), la Asociación Internacional de Probióticos (IPA) y la Sociedad Española de Microbiota, Probióticos y Prebióticos (SEMiPyP).

La relevancia moderna y la revisión de la definición del término probiótico se llevó a cabo en 2013, cuando la Asociación Científica Internacional de Probióticos y Prebióticos (ISAPP) organizó una consulta de expertos clínicos y científicos (Sanders et al., 2018). Como resultado, fue emitida por consenso la definición actual de los probióticos: "son microorganismos vivos que, cuando se administran en cantidades adecuadas, confieren un beneficio para la salud del huésped" (Hill et al., 2014).

Una evaluación más amplia de la seguridad de los probióticos concluyó que estos eran seguros para su uso en personas sanas. Esta conclusión se ve reforzada por la Autoridad Europea de Seguridad Alimentaria (EFSA), que considera que todas las especies comunes de probióticos son seguras para la población en general (Sanders et al. 2016). Muchas cepas probióticas comúnmente explotadas y actualmente disponibles se benefician de un estado generalmente reconocido como seguro (GRAS) en EE. UU. o pertenecen a especies con estatus de presunción de seguridad calificada (QPS) por la Autoridad Europea de Seguridad Alimentaria (EFSA) (Cunningham et al., 2021).

Los probióticos se aíslan en la mayoría de casos tanto de los alimentos fermentados como de la microbiota intestinal humana (Chaudhari y Dwivedi, 2022). Los alimentos fermentados tradicionales contienen microorganismos que pueden haber sido agregados como cultivo para iniciar la fermentación o estar presentes en el material de partida. Las materias primas susceptibles de ser fermentadas y convertidas en alimentos probióticos incluyen verduras, frutas y sus zumos, granos/cereales, productos lácteos, carne, pescado y miel (Cunningham et al., 2021).

La diferencia entre los microorganismos probióticos y los asociados a la fermentación radica en el hecho de que, para considerarse probióticos, los microorganismos tienen que producir un efecto beneficioso sobre la salud, demostrado por lo menos a través de un ensayo positivo en humanos (Sanders et al., 2018; Binda et al., 2020). Pero se podría añadir que, no todos los alimentos fermentados son alimentos probióticos, ya que los productos no definidos y no estudiados no cumplen con los requisitos mínimos exigidos en un probiótico. Sin embargo, en la medida en que se agregue un probiótico a un alimento fermentado o se incluya en la producción de un alimento fermentado, ese alimento fermentado también sería un alimento probiótico (Sanders et al., 2018). El uso de probióticos seleccionados como cultivos iniciadores en procesos tradicionales, sobre todo en aquellos que se llevan a cabo siguiendo la fermentación espontánea, aumenta la seguridad de estos alimentos y ayuda a obtener productos de mayor calidad y homogeneidad en sus propiedades (Sanders et al., 2018).

Por otra parte, están los prebióticos que fueron definidos por primera vez en 1995 por Glenn Gibson y Marcel Roberfroid como “cualquier componente alimentario no viable que confiere un beneficio para la salud del huésped asociado con la modulación de la microbiota” (Gibson y Roberfroid, 1995). Más tarde, en 2017, expertos de la ISAPP definieron los prebióticos como “sustratos que son utilizados selectivamente por los microorganismos beneficiosos albergados por el huésped” incluidas las cepas probióticas administradas y los microorganismos autóctonos (Gibson et al., 2017). A este grupo pertenecen hidratos de carbono tales como los β-fructanos de cadena corta y larga fructooligosacáridos (FOS) e inulina, la lactulosa y los galacto-oligosacáridos (GOS). También los polifenoles y los ácidos grasos poliinsaturados, convertidos en sus respectivos ácidos grasos conjugados, junto con algunos péptidos catabolizados por bacterias en ingredientes activos se incluyen en esta categoría. Por otra parte, existe una variedad de micronutrientes, orgánicos e inorgánicos, que son necesarios para el desarrollo de las bacterias, no solo en el tracto gastrointestinal (TGI), sino en todos los nichos del cuerpo, incluyendo la piel, el tracto urinario y la vagina (Gibson et al., 2017; Pyle, 2021).

Los prebióticos han ganado mucha atención en los últimos años ya que brindan beneficios nutricionales junto con beneficios fisiológicos para la salud (Chaudhari y Dwivedi, 2022). Hasta ahora se ha demostrado que los prebióticos son beneficiosos para el tracto gastrointestinal (por inhibición de patógenos o estimulación de la respuesta inmune), el metabolismo cardiovascular (por reducción de los niveles de lípidos en sangre o por su efecto sobre la resistencia a la insulina), la salud mental (por su influencia en la función cerebral, energía y cognición), los huesos (al aumentar la biodisponibilidad de minerales), la piel (por hidratación de las capas superficiales de la piel y normalizar su queratinización y exfoliación) y el tracto urogenital (reducen el nivel de toxinas en enfermedades renales crónicas). También juegan un papel importante en la reducción de enfermedades metabólicas tales como la obesidad, la diabetes tipo 1 y tipo 2 o la enfermedad del hígado graso no alcohólico.

En el año 2019 la ISAPP convocó un panel de expertos científicos y académicos para brindar claridad y orientación sobre el uso apropiado del término "sinbiótico", que pasó a definirse como "una mezcla que comprende microorganismos vivos y sustrato(s) utilizados selectivamente por éstos y que confiere un beneficio para la salud del huésped” (Swanson et al., 2020). En los sinbióticos, los probióticos utilizan los prebióticos como fuente de alimento, lo que les permite sobrevivir durante un periodo prolongado dentro del intestino. La mejora en la viabilidad de los probióticos favorece que puedan ejercer un beneficio para la salud (Chaudhari y Dwivedi, 2022).

I.1.2. Características de los probióticos

La mayoría de los probióticos se caracterizan por ser microorganismos auxótrofos que no son capaces de sintetizar todos los factores de crecimiento, como las bases nitrogenadas, aminoácidos y vitaminas del complejo B. Forman parte de la microbiota de la leche, la carne, los vegetales, las frutas, los vinos, las mucosas intestinales y vaginales, de donde toman sus requerimientos nutricionales. Son genéticamente

estables, capaces de alcanzar el intestino humano y multiplicarse sin producir daños al huésped (Sanders et al., 2018). Además de por la presencia de nutrientes, el crecimiento de los microorganismos probióticos se ve afectado por el pH y la temperatura del medio, por lo que resulta más fácil incluirlos en unas matrices alimentarias que en otras. Antes de ser añadidos en las matrices alimentarias como materia prima, las cepas específicas de microorganismos tendrán que cumplir unos criterios que permiten decidir si se califican como probióticos para su uso en alimentos y suplementos dietéticos. Tal como detallan Binda et al. (2020), las cepas probióticas deben:

- Estar suficientemente caracterizadas.
- Ser seguras para el uso previsto.
- Ejercer un efecto beneficioso para la salud respaldado al menos por un ensayo clínico positivo en humanos realizado de acuerdo con los estándares científicos generalmente aceptados.
- Permanecer vivas en cantidades suficientes en el producto a una dosis eficaz durante toda la vida útil del mismo.

Según Rondon et al. (2015), para poder considerar y utilizar un microorganismo como probiótico es necesario que reúna una serie de características de seguridad, funcionales y tecnológicas.

1. Requerimientos de seguridad:
    - Las cepas para uso humano deben de ser de origen humano y preferentemente aisladas de humanos sanos.
    - No patógenas ni tóxicas.
    - No portar genes transmisibles de resistencia a antibióticos.
2. Requerimientos funcionales:
    - Supervivencia a las condiciones del ambiente gastrointestinal.
    - Adhesión a superficies epiteliales y persistencia en el tracto gastrointestinal.
    - Inmunoestimulación, pero sin efecto proinflamatorio.
    - Actividad antagonista contra patógenos.
    - Propiedades antimutagénicas y anticarcinogénicas.
3. Requerimientos tecnológicos:
    - Contener un número adecuado de cepas viables que conduzcan al efecto beneficioso demostrado.
    - Resistencia a fagos.
    - Viabilidad durante el procesado y el almacenamiento.
    - Estabilidad en el producto y durante el almacenamiento.

Otra característica de los microorganismos probióticos es que modifican la composición química del alimento durante la fermentación y, en consecuencia, alteran sus propiedades nutricionales (Sanders et al., 2018).

Las especies de *Lactobacillus y Bifidobacterium* son las más utilizadas como probióticos, pero también se utiliza la levadura *Saccharomyces boulardii* y otros microorganismos de las siguientes especies: *Bacillus, Propionibacterium, Streptococcus y Escherichia*. El

último microorganismo aprobado recientemente y añadido en esta lista es *Clostridium butyricum* (*Nueva edición de la Guía de Probióticos y Prebióticos de la WGO|El Probiótico n.*d.). En la Tabla 1 están representados los principales microorganismos utilizados como probióticos en actualidad.

Tabla 1. Microorganismos vigentes utilizados como probióticos.
Fuente: *(*Chaudhari y Dwivedi, 2022*)*

Probiotic genus	Species
Akkermansia	*Akkermansia muciniphila*
Bacillus	*Bacillus coagulans, Bacillus subtilis, Bacillus laterosporus*
Bacteroides	*Bacteroides uniformis*
Bifidobacterium	*Bifidobacterium breve, Bifidobacterium adolescentis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifiobacterium infantis, Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium catenulatum, Bifidobacterium thermophilum*
Enterococcus	*Enterococcus faecium, Enterococcus faecalis*
Lactobacillus	*Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus reuteri, Lactobacillus delbrueckii ssp. (Lactobacillus bulgaricus), Lactobacillus fermentum, Lactobacillus johnsonii, Lactobacillus brevis, Lactobacillus curvatus, Lactobacillus lactis, Lactobacillus cellobiosus*
Leuconostoc	*Leuconostoc lactis subsp. cremoris, Leuconostoc mesenteroides*
Pediococcus	*Pediococcus acidilactici*
Peptostreptococcus	*Peptostreptococcus productus*
Propionibacterium	*Propionibacterium jensenii, Propionibacterium freudenreichii*
Saccharomyces	*Saccharomyces cerevisiae Saccharomyces boulardii*
Streptococcus	*Streptococcus oralis, Streptococcus uberis, Streptococcus rattus, Streptococcus salivarius, Streptococcus sanguis, Streptococcus intermedius Streptococcus mitis, Streptococcus thermophilus, Streptococcus diacetylactis, Streptococcus cremoris,*

Lactobacillus salivarius es una bacteria gram positiva homofermentativa que produce solo ácido láctico a partir del metabolismo de los carbohidratos. Es una bacteria del ácido láctico (BAL) con el estatus de Presunción Cualificada de Seguridad (QPS) por la Autoridad Europea de Seguridad Alimentaria (Panel de la EFSA sobre Peligros Biológicos) que se encuentra naturalmente en el tracto humano, la leche humana, vagina humana y cavidad oral humana, entre otras fuentes. En los últimos años, *L. salivarius* ha estado ganando atención como un microorganismo probiótico prometedor con un número creciente de estudios que exploran una amplia variedad de aplicaciones (Guerrero-Sánchez et al., 2021).

Hay evidencias de que *L. salivarius* spp. *salivarius* coloniza el estómago y produce factores inmunomoduladores que suprimen la inflamación causada por la infección por *H. pylori* de las células epiteliales gástricas. En este caso, será necesario que *L. salivarius* spp. *salivarius* mantenga su forma activa hasta el estómago, donde podrá competir con

la bacteria *Helicobacter pylori* e interactuar con el tejido epitelial gástrico para ejercer un efecto positivo contra la infección (Aiba, 1998).

I.1.3. Mecanismo de acción de los probióticos

La mayoría de los probióticos con efectos probados sobre la salud actúan a nivel del tracto gastrointestinal (TGI). El TGI es un ecosistema complejo colonizado por multitud de microbios aproximadamente 1000 especies de anaerobios facultativos o estrictos, cuya composición y actividad son esenciales para la salud humana (Kerry et al., 2018; Kwofie et al., 2020).

A pesar de la existencia de mecanismos generales y compartidos para las funciones probióticas, no todas las cepas probióticas son iguales (Sanders et al., 2018). Es muy importante que cada cepa se pruebe por sí sola o en productos diseñados para una función específica. A través de las nuevas técnicas moleculares se pueden investigar los probióticos y obtener las propiedades específicas de cada cepa (Nagpal et al., 2012). La tendencia futura es que estos efectos dependan no solo de cada cepa bacteriana, sino también de la dosis utilizada (Hussein, 2022).

Según Sarao y Arora (2017), los mecanismos de acción de los probióticos en el TGI se pueden explicar teniendo en cuenta si actúan a nivel luminal, de la mucosa o de la submucosa:

- El efecto luminal básico de los probióticos aparece en la luz intestinal y mejora el equilibrio microbiano intestinal. A través del consumo de probióticos se mantiene o promueve la homeostasis del TGI y se ha encontrado que los probióticos estimulan el crecimiento de microbios intestinales beneficiosos autóctonos como las bifidobacterias e inhiben el crecimiento de microbios patógenos u oportunistas.
- A nivel de mucosa intestinal, el efecto de los probióticos incluye un aumento en la producción de mucina del huésped, lo que mejora la capacidad de la capa de moco para actuar como un escudo antibacteriano.
- Los probióticos actúan a nivel de submucosa sobre el sistema inmunitario del huésped mejorando las funciones de la barrera inmunológica del intestino y aliviando la respuesta inflamatoria intestinal mediante mecanismos que incluyen diversos efectos sobre la activación inmunitaria, la producción de citoquinas, la inmunomodulación y la inflamación.

Algunos mecanismos pueden estar muy extendidos entre los géneros de probióticos comúnmente estudiados, observarse con frecuencia entre la mayoría de las cepas de una especie probiótica, o ser raros y estar presentes solo en unas pocas cepas de una especie determinada. A continuación, se detallan algunos de estos mecanismos (Hill et al., 2014; Sanders et al., 2018).

- En primer lugar, están los mecanismos comunes a diferentes géneros de probioticos tales como la resistencia a la colonización, la producción de ácidos

grasos de cadena corta (AGCC), la regulación de la barrera intestinal, la exclusión competitiva de patógenos.

- En segundo lugar, están los mecanismos propios de una especie tales como la síntesis de vitaminas, el antagonismo directo, reforzar la barrera intestinal, la actividad enzimática, el metabolismo de las sales biliares y la neutralización de los carcinógenos.

  Por último, los mecanismos que son más recientes y específicos de una cepa en concreto están relacionados con los efectos neurológicos, inmunológicos, endocrinológicos y la producción de compuestos bioactivos.

I.1.4. Efectos beneficiosos de los probióticos

Tanto el panel de consenso de ISAPP como la Sociedad Española de la Microbiota Probióticos y Prebióticos (SEMiPyP) abordaron la evolución del concepto de especificidad de la cepa del probiótico y los efectos beneficiosos sobre la salud y mostraron que varios mecanismos probióticos son responsables de ciertos beneficios que pueden ser compartidos comúnmente entre la mayoría de las cepas de un grupo taxonómico más grande (Sanders et al., 2018).

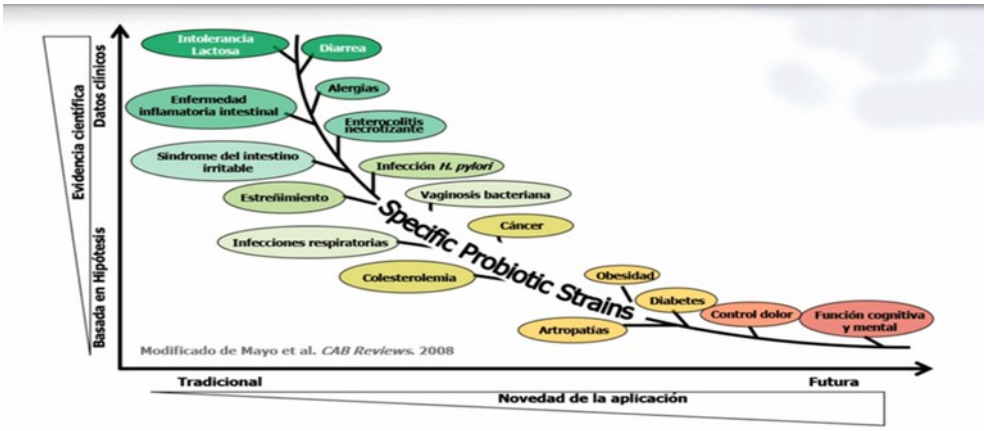

Figura 2. Efectos beneficiosos de los probióticos sobre la salud. Fuente: Sociedad Española de la Microbiota Probióticos y Prebióticos (SEMiPyP, 2021).

Tal y como se muestra en la Figura 2, SEMiPyP detalla que hay una relación directamente proporcional entre la cantidad de datos clínicos que aumentan la evidencia científica y que demuestran los efectos beneficiosos sobre la salud de cepas especificas de probióticos en el tiempo. En el caso del yogur, por ejemplo, la Autoridad Europea de Seguridad Alimentaria (EFSA) aprobó en 2010 una declaración de propiedades saludables para este alimento fermentado probiótico, al haber constatado que las bacterias presentes (*Streptococcus thermophilus* y *Lactobacillus delbrueckii* subsp. *bulgaricus*) ayudan a mejorar la digestión de la lactosa en personas con intolerancia a este disacárido (Sanders et al., 2018).

A continuación en la Tabla 2, se presentan en detalle la relación entre las cepas de probióticos y los efectos sobre la salud constatados a través de estudios científicos con animales o en humanos.

Tabla 2. Efectos beneficiosos sobre la salud producidos por diferentes cepas de microorganismos probióticos.

Microorganismo probiótico	Efectos beneficiosos sobre la salud	Referencia
Lactobacillus plantarum MONO3	Fortalecer el sistema inmunitario	(Ashaolu, 2020)
Streptococcus thermophilus y Lactobacillus delbrueckii spp. *bulgaricus*	Intolerancia a lactosa	(Bultosa, 2016; Sanders et al., 2018)
Bifidobacterium longum 536	Enfermedad inflamatoria intestinal (colita ulcerativa)	(Pyle, 2021)
Lactobacillus rhamnosus GG	Alergias	(Pyle, 2021)
Lactobacillus acidophilus NCFM	Síndrome Intestino Irritable (SII)	(Pyle, 2021)
Bifidobacterium breve M16V	Enterocolitis necrotizante	(Pyle, 2021)
Lactobacillus rhamnosus GG	Estreñimiento	(Pyle, 2021)
Lactobacillus salivarius Bifidobacterium animalis ssp. *lactis* B94	Infección *Helicobacter pylori*	(Aiba 1998) (Pyle, 2021)
Bifidobacterium animalis ssp. *lactis* BB12	Infecciones respiratorios	(Pyle, 2021)
L. rhamnosus GR-1 *and L. reuteri* RC-14	Vaginosis bacteriana	(Adesulu-Dahunsi, 2022)
Lactobacillus casei SHIROTA *Lactobacillus rhamnosus* 231 *Lactobacillus acidophillus*	Cáncer	(Lee et al., 2022)
Lactobacillus plantarum YS 5	Colesterolemia	(Roobab et al., 2020)
Bifidobacterium animalis ssp. *lactis* Ba82145	Obesidad	(Pyle, 2021)
Bifidobacterium lactis HN019	Diabetes	(Pyle, 2021)
Citrobacter rodentium; Prevotella; Collinsella aerofaciens	Artropatías	(Kalinkovich y Livshits 2019)
Lactobacillus rhamnosus (LGG®), Saccharomyces cerevisiae (boulardii) Bifidobacterium animalis ssp *lactis.*	Control del dolor	(Taye et al., 2020)
Bifidobacterium longum 1714	Función cognitiva y mental	(Pyle, 2021)

I.1.5. Alimentos funcionales

El concepto de "alimento funcional" fue introducido por primera vez en 1991 por el gobierno japonés a través de los alimentos FOSHU (Foods for Specific Health Use) para referirse a alimentos enriquecidos con ingredientes tales como vitaminas, proteínas, fibra, bacterias probióticas u otros aditivos alimentarios que pueden contribuir a la salud humana y al bienestar (Min et al., 2019). A principios de 1996, la Comisión Europea, mediante la Acción Concertada en Alimentos Funcionales en Europa (FUFOSE), llegó a un consenso sobre la definición de alimento funcional (Roberfroid, 2000). Se estableció que "un alimento puede considerarse funcional si se demuestra satisfactoriamente que afecta de manera beneficiosa a una o más funciones específicas del organismo, más allá de los efectos nutricionales, de manera que mejore la salud y/o ayude a reducir el riesgo de enfermedad, además de tener la apariencia de un alimento convencional (Ashaolu, 2020).

La ISAPP restringió el uso del término probiótico solo en productos que entregan microorganismos seguros y vivos con recuentos viables adecuados 10^6 UFC/g de producto de cepas bien definidas con una expectativa razonable de entregar beneficios para la salud al anfitrión (Bultosa, 2016; Terpou et al., 2019). Diferentes investigaciones han demostrado la factibilidad de utilizar diferentes matrices alimentarias para la incorporación de probioticos. Tanto los alimentos probióticos lácteos como los no lácteos han estado desde la antigüedad presentes en la alimentación de la población a través del consumo de leches fermentadas y de otros alimentos fermentados, como frutas, hortalizas y productos cárnicos.

En los alimentos probióticos de origen lácteo como yogur, queso, helados, cuajada, leche fermentada, etc., la composición físico-química de la matriz láctea, rica en proteínas y lípidos, confiere cierta protección a los probióticos y aumenta su resistencia durante el paso por el TGI hasta que lleguen al sitio de acción en el intestino delgado (Vijaya Kumar et al., 2015). En relación con esto, Roobab et al. (2020) constataron una mayor supervivencia de las cepas probióticas de *L. acidophilus* y del género *Bifidobacteria* a su paso por el TGI cuando se ingieren como parte de una leche fermentada que cuando se ingieren en forma de suplementos dietéticos, lo que demuestra que la composición de la leche mejora la capacidad de los probióticos para tolerar un pH bajo y la acción de las enzimas.

Sin embargo, los productos lácteos no son aptos para veganos y vegetarianos, contienen alérgenos y presentan un elevado contenido en colesterol, por lo que no pueden ser ingeridos por consumidores que padecen enfermedades cardiovasculares u obesidad. Ante esta situación, los alimentos probióticos de origen vegetal ofrecen características y ventajas únicas como alternativas a los alimentos probióticos lácteos. Además, tal como detalla Nagpal et al. (2012), la incorporación de probióticos en este tipo de matrices tiene varias ventajas como:

- Mejora las propiedades sensoriales, como el aroma de los productos de soja.
- Previene e inhibe el deterioro y el crecimiento de patógenos.
- Suprime factores anti-nutricionales.
- Aumenta el contenido en antioxidantes.

Se ha observado durante la pandemia ocasionada por el virus Sars-Cov2 un incremento de la demanda de ingredientes alimentarios que ayuden a fortalecer el sistema inmune. Los alimentos probióticos pueden contribuir a mejorar la respuesta inmunitaria, lo que proporciona defensas al huésped contra las infecciones y suprime las alergias y la inflamación (Ashaolu, 2020; Pimentel et al., 2021).

Teniendo en cuenta que uno de los desafíos clave que se presenta en el siglo XXI es la necesidad de alimentar a una población humana cada vez mayor con recursos naturales limitados (Pimentel et al., 2021), las nuevas formas de alimentos funcionales deben adaptarse a las necesidades globales y futuras. Estas necesidades globales quedaron establecidas en la Agenda 2030 que cuenta con 17 Objetos de Desarrollo Sostenible (ODS) y que fue aprobada por la Asamblea General de la ONU en 2015. Concretamente

los ODS 2 y 3 abogan por erradicar el hambre y la malnutrición en todas sus formas, desnutrición y obesidad. Adicionalmente, el ODS 12 aboga por formas de producción y consumo más sostenibles. En este sentido se favorece el aprovechamiento y desarrollo de alimentos de origen vegetal frente a los de origen animal. Estos 3 objetivos ya están condicionando y, con toda seguridad, condicionarán la investigación de los próximos años en el área de los alimentos funcionales y, dentro de estos, la de aquellos con probióticos.

Se estima que aproximadamente 1 de cada 9 personas en el mundo está desnutrida, principalmente debido a la desnutrición proteico-energética por este motivo se ha fijado el ODS número 2: hambre cero para el 2030. Para llevarlo a cabo, la comunidad científica aumentó la investigación de nuevos componentes naturales y el desarrollo de nuevos productos, incrementando la innovación en el sector alimentario y la creación de nuevos nichos de mercado, principalmente relacionados con los alimentos funcionales.

El ODS 3 tiene como meta garantizar una vida sana y promover el bienestar en todas las edades es esencial para el desarrollo sostenible. Actualmente, el mundo se enfrenta a una crisis sanitaria mundial sin precedentes debido a COVID-19 y también a un conflicto armado en Europa. Estos eventos propagaron el sufrimiento humano, desestabilizaron la economía mundial y cambiando drásticamente las vidas de miles de millones de personas en todo el mundo.

Algunas de las tendencias que se observan en esta línea son:

- Aprovechamiento de materias primas autóctonas y fortalecimiento de la soberanía alimentaria. En este sentido, los alimentos tradicionales fermentados propios de comunidades locales constituyen una enorme oportunidad para la identificación de nuevos probióticos.
- Nuevas fuentes de proteína. La inclusión de determinados microrganismos puede resultar una fuente de proteína importante.
- Aprovechamiento de técnicas sostenibles que favorezcan el desarrollo de alimentos más nutritivos y con componentes de interés.

En este sentido la comunidad científica ha mantenido su interés en investigar y descubrir nuevos alimentos para el mantenimiento de la salud llevándose a cabo una multitud de estudios que han demostrado que algunos alimentos funcionales reducen el riesgo de padecer ciertas enfermedades (Pimentel et al., 2021).

I.1.6. Estudios que demuestran los efectos sobre la salud de los alimentos probióticos con matriz vegetal

La eficacia de los probióticos está asociada con su viabilidad en los productos alimenticios, y se ha afirmado que varios factores son responsables de su reducción. En este sentido se llevan a cabo varios enfoques para mejorar y mantener la viabilidad de las células microbianas, como la selección adecuada de probióticos y elegir las matrices

alimentarias adecuadas. Otra forma de estimular el crecimiento de los probióticos, que está en auge, es el desarrollo de las combinaciones sinbióticas (Sengupta et al., 2019) .

Porque contienen nutrientes fácilmente asimilables, se podrían elegir alimentos con matriz vegetal. Las frutas y las verduras, además de las legumbres y los cereales, pueden ser empleados como substrato para bacterias probióticas. Los probióticos que mejor se adecúan a este tipo de alimentos pertenecen a los géneros *Lactobacillu*s y *Bifidobacteria,* fundamentalmente.

En la composición de estos alimentos se encuentran xylo-oligosacaridos, galacto-oligosacaridos, polydextrosa, fenoles y antioxidantes que actúan como prebióticos y proporcionan nutrientes que estimulan el crecimiento y la actividad de la microbiota intestinal (Min et al., 2019). En la actualidad existe una gran variedad de vegetales que se emplean como matriz vegetal para el crecimiento probiótico (zumos de frutas, frutas mínimamente procesadas, vegetales fermentados, snacks, aceitunas fermentadas, bebidas de cereales fermentados, leches fermentadas enriquecidas con fruta, legumbres fermentadas) y que además tienen la ventaja de no contener colesterol ni lactosa, ser aptos para veganos y aportar dosis considerables de antioxidantes. Por lo general, las bebidas son las matrices con mejores índices de crecimiento tanto en procesado como en conservación, además de las mejor aceptadas por la población, pese a que la adición de probióticos en zumos de frutas y verduras proporciona aromas desagradables (Misra et al., 2021).

Una vez elegida la matriz vegetal que podría facilitar el crecimiento del probiótico, la siguiente etapa y condición importante para todos los nuevos alimentos funcionales, antes de ser lanzados al mercado, es el análisis de simulación gastrointestinal (SGI) *in vitro* para comprobar la adecuada biodisponibilidad de los compuestos de interés (Rodríguez-Roque et al., 2013), la cual se puede ver influenciada por su interacción con otros ingredientes presentes en el alimento, por las operaciones unitarias aplicadas durante el procesado del alimento, por el tipo de estructura que presente y el grado de protección que les confiera la matriz alimentaria (Madureira et al., 2011).

Para llevar a cabo este tipo de ensayos se pueden utilizar modelos experimentales *in vivo*, *in vitro* e *in sillico*. Se entiende por experimento *in vivo* aquel que se lleva a cabo con especies animales y ex *vivo* en humanos. Los experimentos *in vitro* son realizados en dispositivos de laboratorio utilizando tejidos, células o moléculas provenientes de las especies animales. Por último, los experimentos *in sillico* son simulaciones o reproducciones de experimentos *in vivo* o *in vitro*, que emplean modelos matemáticos y softwares de simulación.

Se ha constatado que los alimentos probióticos de origen vegetal pueden mejorar el perfil lipídico y el sistema inmunológico, ayudan en el control de la diabetes, reducen el riesgo de infección por *Helicobacter pylori* y enfermedades asociadas, ayudar a prevenir el cáncer y, de forma general, contribuyen al bienestar general, estos resultados siendo obtenidos a través de estudios *in vitro* e *in vivo* (Ashaolu, 2020). En este sentido en la Tabla 3 se detallan algunos estudios *in vivo* de los alimentos con matriz vegetal

inoculados con probióticos que demuestran parte de los efectos beneficiosos sobre la salud arriba presentados.

Se puede concluir que los alimentos probióticos con matriz vegetal se presentan como el desafío industrial más novedoso, para cubrir un nicho de mercado creciente y muy prometedor que claramente exige condiciones estrictas para sus productos (Pimentel et al., 2021).

Tabla 3 -Estudios in vivo que demuestran los efectos beneficiosos sobre la salud de los alimentos con matriz vegetal inoculados con probióticos. Fuente (Pimentel et al., 2021)

Nº.	Objetivo	Cepa utilizada	Procesamiento	Análisis	Beneficios sobre la salud
1.	Producir una bebida fermentada con orujo de arándanos optimizando la cepa y la tecnología de procesamiento	*Lacticaseibacillus rhamnosus GG (antiguo Lactobacillus rhamnosus GG), Lactiplantibacillus plantarum-1 (antiguo Lactobacillus plantarum-1) y Lactiplantibacillus plantarum-2 (antiguo L. plantarum-2).*	Se agregaron cepas individuales de *Lactobacillus* y cepas mixtas 1:1 (v: v) al líquido de orujo de arándanos con una concentración de 7,5 log UFC/mL.	Medición de la actividad antifatiga mediante prueba de natación con carga de peso en ratones. Los animales que consumieron el producto probiótico tuvieron un tiempo de nado 4 veces mayor que los animales que consumieron agua esterilizada y también perdieron mayor peso.	El tiempo de natación del grupo probiótico fue similar al de los animales que consumieron Red Bull, una famosa bebida antifatiga. Por lo tanto, la bebida fermentada probiótica podría ser un nuevo producto antioxidante y antifatiga.
2.	Desarrollar un yogur de soja simbiótico que contenga una cápsula sinbiótico LactoBacil Plus para investigar los efectos en los perfiles de lípidos plasmáticos de la colección de cultivos tipo microbianos y el banco de genes (Chandigarh).	*Lactobacillus acidophilus y Lacticaseibacillus rhamnosus (antiguo Lactobacillus rhamnosus), Bifidobacterium longum, B. bifidum y Saccharomyces boulardii y fructooligosacárido.*	Leche de soja inoculada con 2 g de cápsulas de gelatina dura de SCLBP (cápsula sinbiótica LactoBacil Plus que contiene *Lactobacillus acidophilu*s y *Lacticaseibacillus rhamnosus* (antiguo *Lactobacillus rhamnosus), Bifidobacterium longum, B. bifidum y Saccharomyces boulardii* y fructooligosacárido	Análisis bioquímicos de suero, ensayo de enzimas hepáticas en suero sanguíneo y evaluación del estrés oxidativo de ratones alimentados con diferentes dietas experimentales.	Los yogures tuvieron un efecto favorable en la reducción del colesterol en sangre. Condujeron a una disminución significativa del índice aterogénico en comparación con el yogur de soja (control) solo. El tratamiento con cultivos sinbióticos de yogur de soja mejora la peroxidación lipídica en el hígado.
3.	Desarrollar jugo de litchi probiótico fermentado por *Lacticaseibacillus casei* (antiguo *Lactobacillus casei)* y evaluar sus efectos sobre la función inmunomoduladora y el intestino	*Lacticaseibacillus casei (antiguo Lactobacillus casei*)	El jugo de litchi fue fermentado por *L. casei* para producir un contenido inicial de 10^6 UFC/mL.	Determinación en ratones: índices de timo y bazo, examen histopatológico y microbiológico.	El jugo de litchi fermentado con probiótico podría mejorar la actividad inmunomoduladora de los ratones al mejorar los índices de los órganos inmunitarios (bazo, timo), estimular las secreciones de citoquinas, inmunoglobulinas y proteger el tracto intestinal. Se observó un aumento significativo de las bacterias beneficiosas en la microbiota (*Faecalibaculum, Lactobacillus y Akkermansia*)

4.	Investigar los efectos de la mezcla de cereales fermentados con el probiótico *Pichia kudriavzevi*i OG32 sobre los niveles de marcadores antioxidantes en plasma en ratas	*Pichia kudriavzevii OG32*	El alimento funcional a base de cereales se preparó a partir de granos enteros de una mezcla de cereales limpios, incluidos sorgo blanco y rojo, mijo perla, trigo y se fermentó con *Pichia kudriavzevii* OG32.	Determinación de: Marcadores bioquímicos y antioxidantes en plasma; Perfil lipídico sérico; Perfil lipídico hepático.	El contenido de colesterol total (TC), triacilglicerol (TG) y colesterol LDL de ratas alimentadas con una dieta alta en colesterol disminuyó. Además, se observó una reducción del índice aterogénico.
5.	Evaluar la viabilidad de *Lacticaseibacillus rhamnosus* GG (antiguo L*actobacillus rhamnosus* GG) en jugo mixto de piña fermentada y jussara cuando se somete su resistencia *in vitro* e *in vivo* al tracto gastrointestinal (TGI).	*Lacticaseibacillus rhamnosus GG (antiguo Lactobacillus rhamnosus GG)*	El jugo se formuló con un 50 % de pulpa de ananá, un 45 % de pulpa de jussara y un 5,0 % de sacarosa. Probiótico de concentración 10^{10} LGG (Culturelle®) en 200 mL del jugo enfriado.	Efecto de la ingesta del jugo probiótico sobre el desarrollo de lesiones preneoplásicas en ratas Wistar: Evaluación bioquímica de la sangre. Determinación de ácidos grasos de cadena corta y valor de pH fecal.	Sin hepatotoxicidad ni nefrotoxicidad según los valores de referencia para ratas Wistar, lo que demuestra la seguridad. Contribuyó a la reducción de la fracción de colesterol LDL, lo que ayudó a prevenir la enfermedad coronaria.
6.	Investigar el efecto del jugo de zanahoria fermentado con *Lactiplantibacillus plantarum* NCU116 (anteriormente *Lactobacillus plantarum* NCU116) sobre los metabolitos del suero de ratas diabéticas tipo 2 inducidas por estreptozotocina y con una dieta rica en grasas.	*Lactiplantibacillus plantarum NCU116 (anteriormente Lactobacillus plantarum NCU116)*	El jugo de zanahoria se inoculó al 4% (v/v) con el fermento que contenía ≈ 9 log UFC/mL del probiótico *L. plantarum* NCU1160.	Análisis de metabolitos de muestras de suero de ratas diabéticas tipo 2 inducidas con dosis bajas de estreptozotocina y dieta alta en grasas.	Cambios positivos en las rutas y procesos biológicos, como el metabolismo de lípidos, lipoproteínas, purina, triptófano, secreción de bilis, biosíntesis de ácidos grasos, glucólisis y gluconeogénesis de la diabetes tipo 2 en ratas. Mejora de la diabetes tipo 2 en ratas.
7.	Investigar si el jugo de cítricos fermentado podría aliviar los síntomas de la rinitis alérgica perenne en un estudio paralelo doble ciego, controlado con placebo.	*Lactiplantibacillus plantarum LP0132 (antiguo Lactobacillus plantarum LP0132)*	El jugo de cítricos fue fermentado con *Lactiplantibacillus plantarum* LP0132 (antiguo *Lactobacillus plantarum* LP0132), a 37 ◦C por 48 h, y esterilizado por calor a 90 ◦C por 5 min.	En los enfermos con rinitis alérgica los síntomas se examinaron a través de cuestionarios que constaban de cuatro ítems (estornudos, rinorrea, congestión nasal y dificultad en las rutinas diarias).	Reducción significativa en la puntuación de los síntomas nasales y la puntuación de la congestión nasal durante el período de intervención.
8.	Descubrir los efectos de la leche de soja probiótica a los marcadores de la metilación MLH1 y MSH2 y el estrés oxidativo en pacientes con diabetes tipo II.	*Lactiplantibacillus plantarum A7 (antiguo Lactobacillus plantarum A7*)	Los pacientes con diabetes mellitus tipo II fueron asignados a dos grupos en este ensayo clínico aleatorizado, doble ciego y controlado.	A los dos grupos se les han tomado muestras de sangre en ayuno para medir la actividad de 8-hidroxi-20-desoxiguanosina (8-OHdG) y superóxido dismutasa (SOD); Se	La leche de soya probiótica disminuyó significativamente la metilación del promotor MLH1, mientras que la concentración plasmática de 8-OHdG disminuyó significativamente en comparación con la leche de soya. Se

			Grupo de intervención: consumió 200 mL/día de leche de soja probiótica que contenía *Lactiplantibacillus plantarum* A7 (antiguo *Lactobacillus plantarum* A7) (contenía 7,3 log UFC/mL); Grupo control: consumió 200 mL/día de leche de soja convencional durante 8 semanas.	recogieron también las medidas antropométricas (peso, altura de pie, índice de masa corporal) y recordatorios dietéticos de 24 horas al inicio del estudio.	observó un aumento significativo en la actividad de SOD y no hubo cambios significativos desde el inicio en la metilación del promotor de MSH2 dentro de ninguno de los grupos. El consumo de leche de soya probiótica mejoró el estado antioxidante en pacientes diabéticos tipo II.
9.	Estudio de los efectos de ingerir una bebida sinbiótica a base de yacón (fuente prebiótica) y extractos de soya, que contienen probióticos. *Bifidobacterium animalis ssp. lactis* BB-12 en la concentración de poliaminas fecales de personas mayores.	*Bifidobacterium animalis ssp. Lactis*(BB-12®)	La base estándar constaba de 60 % de extracto de soja, 40 % de extracto de yacón, 0,14 % de estabilizador y 0,3 % de gelatina en polvo. Cultivo probiótico: BB-12®-Cultivo-probiótico-Probio-Tec® (o log UFC/100 mL de producto). El tiempo total del experimento fue de 8 semanas, que se dividió en 3 fases consecutivas (prealimentación, alimentación y postalimentación).	Estudio doble ciego en el que las personas mayores voluntarias se dividieron aleatoriamente en dos grupos que recibieron bebidas simbióticas o placebo. Análisis: Determinación de la concentración de poliaminas fecales; Enumeración de evaluación de bacterias fecales.	El consumo de bebidas sinbióticas o placebo no indujo ningún efecto significativo sobre la producción de citocinas proinflamatorias, factor de necrosis tumoral alfa, interleucina-6 e interleucina-10 antiinflamatoria. Tanto el consumo de bebidas con simbiótico como con placebo han aumentado los niveles de poliaminas, sin influir en las respuestas inflamatorias y parece contribuir al mantener el aumento de poliaminas.

I.2. La ingeniería de los procesos alimentarios aplicada a la mejora de la funcionalidad de los alimentos probióticos

Los alimentos probióticos son susceptibles de sufrir transformaciones físicas, químicas, biológicas cuyos efectos dependerán de factores como el tipo de matriz, las características de la cepa, la dosis utilizada, el pretratamiento o la viabilidad de la cepa, y tendrán impacto en la funcionalidad de los microorganismos. Se requieren tecnologías apropiadas para su procesado, con el fin de obtener una cantidad suficiente de probióticos de forma segura para el consumo (Asaithambi et al., 2021).

Entre las variables que hay que tener en cuenta para poder garantizar el nivel de probióticos en los alimentos destacan la temperatura y la resistencia de la cepa para soportar las condiciones de procesado. En la industria alimentaria la mayoría de las técnicas de pasteurización aplican altas temperatura para erradicar los microorganismos responsables del deterioro presentes en los alimentos. Esto hace que, en alimentos procesados, conservar los microorganismos beneficiosos desde la fabricación hasta el final del tracto gastrointestinal en una concentración mínima de 10^6 UFC/g (Sanz, 2007; Terpou et al., 2019) sea una tarea difícil.

El término bioaccesibilidad hace referencia a la liberación de los compuestos nutritivos presentes en la matriz alimentaria en los jugos digestivos del TGI. Una vez liberados estos compuestos, la proporción que se absorbe y llega efectivamente a la circulación sistémica representa su biodisponibilidad (Betoret et al., 2015). Teniendo en cuenta que los alimentos son en su mayoría mezclas complejas de macro y microcomponentes, organizados en una estructura que puede atrapar compuestos activos, modulando su liberación o inhibiendo su actividad, la matriz alimentaria puede tener una influencia significativa en la actividad o liberación de los componentes clave (Betoret et al., 2015).

Además de las anteriores variables independientes, también se resaltan aquellas asociadas a las condiciones de almacenamiento, como el tipo y material de envasado, las cuales son muy importantes en la calidad final de los alimentos que contienen cultivos probióticos.

En relación con las tecnologías disponibles para la obtención de alimentos con probióticos, podemos decir que desarrollar alimentos probióticos lácteos es relativamente sencillo, pues la propia matriz ejerce un efecto protector; sin embargo, desarrollar alimentos probióticos de origen vegetal es un proceso más complejo que exige una investigación dirigida a que el producto final sea estable, rentable y presente adecuadas propiedades nutricionales y sensoriales (Asaithambi et al., 2021). A pesar de las dificultades técnicas, es posible obtener alimentos probióticos de origen vegetal (snacks, zumos de frutas, vegetales, frutas cortadas) y que estos lleguen a los estantes de los supermercados de todo el mundo. Por ejemplo, en 2014, la empresa estadounidense Ganeden Inc. lanzó el primer zumo de frutas tratado con altas presiones hidrostáticas (HHP) que contenía probióticos (Huang et al., 2017).

Las tecnologías que se van a tratar a continuación han sido elegidas debido a los efectos positivos que producen en la funcionalidad de los alimentos probióticos, permitiendo la

obtención de alimentos funcionales seguros y de calidad. Por una parte, la impregnación a vacío (IV) permite incorporar un líquido en la estructura porosa de una fruta o verdura sin necesidad de someter al producto a una temperatura elevada. Para aumentar el rendimiento del proceso, la homogeneización a alta presión puede mejorar las características tecnológicas y funcionales de los líquidos de impregnación introducidos en la estructura porosa. Finalmente, las tecnologías de deshidratación permiten obtener un producto con una larga vida útil y características estructurales específicas. Adicionalmente, Betoret et al. (2011) mostraron cómo estas técnicas pueden aplicarse de una forma dirigida para promover la formación de estructuras específicas que ayuden a prevenir el deterioro de los compuestos fisiológicamente activos y mantener e incluso aumentar la funcionalidad de los mismos.

I.2.1. Tratamiento por altas presiones

En la industria alimentaria se utilizan dos tecnologías para el tratamiento por altas presiones: alta presión hidrostática (HHP), cuando se aplican presiones entre 150 y 900 MPa y toda la masa recibe durante todo el tiempo de tratamiento la misma intensidad de presión; y la alta presión de homogeneización (HPH), cuando se aplican presiones entre 3 y 500 MPa a líquidos de baja densidad. Ambas se pueden aplicar con el mismo objetivo, pero el principio de acción es diferente. En ambos casos, el nivel de presión, las condiciones del proceso (temperatura, tiempo de reacción, temperatura de entrada y salida, geometría) y las características estructurales de la matriz alimentaria sobre la que se aplican determinan el efecto final (Castagnini et al., 2014).

El proceso de homogeneización a alta presión (HPH) es un proceso tecnológico no térmico, utilizado principalmente para eliminar patógenos e inactivar enzimas y mejorar la calidad nutricional y tecnológica de los productos alimenticios (Betoret et al., 2015). La HPH se aplicó por primera vez en la industria alimentaria para la desinfección de alimentos. Posteriormente, su aplicación se ha dirigido hacia la inactivación de enzimas y, finalmente, los estudios se han centrado en evaluar el efecto de la HPH sobre el contenido en diferentes compuestos bioactivos. Desde 2014 hasta la actualidad, la investigación se centra en la obtención de alimentos funcionales (Castagnini et al., 2014).

La homogeneización a alta presión se consigue al hacer pasar un alimento fluido a través de una válvula, lo que permite alcanzar presiones elevadas, logrando mejorar tanto el aspecto visual como el sabor y el contenido en compuestos bioactivos suspendidos (Castagnini et al., 2014). Algunos estudios han aplicado esta nueva tecnología para aumentar la supervivencia de cepas con efecto probiótico o mejorar sus propiedades funcionales (Patrignani et al., 2009). En ensayos con cepas de *Lactobacillus paracasei A13*, la aplicación de altas presiones aumentó su hidrofobicidad, directamente relacionada con su capacidad de adhesión a las células intestinales y su resistencia al proceso de digestión (Tabanelli et al., 2012).

Con respecto al efecto del tratamiento con altas presiones de homogeneización sobre el contenido en componentes bioactivos con efecto antioxidante, diferentes estudios

demuestran una ventaja en su estabilidad con respecto a la aplicación de tratamientos térmicos. Concretamente en zumo de mandarina, la homogeneización disminuye el tamaño de las partículas suspendidas, aumentando la estabilidad de la nube y, con ello, la disponibilidad de los componentes activos con propiedades antioxidantes (Betoret et al., 2009).

En la actualidad, el uso de la homogeneización a alta presión se ha establecido como una tecnología novedosa no térmica, capaz de mantener, mejorar la calidad y el valor nutricional de los productos alimenticios en comparación con los productos estabilizados mediante tratamientos térmicos. El mecanismo de acción tiene como base la hipótesis según cuál, bajo la influencia de la presión, las moléculas pequeñas, como los compuestos volátiles, los pigmentos, los aminoácidos y las vitaminas, no se ven afectadas debido a que sus estructuras son relativamente simples. En cambio, las moléculas más grandes, como proteínas, enzimas, polisacáridos y ácidos nucleicos, sí que pueden verse afectadas (Balci y Wilbey, 1999).

I.2.2. Impregnación a vacío (IV)

La impregnación a vacío permite, mediante la aplicación de gradientes de presión, la incorporación de componentes a la matriz estructural de los alimentos sin modificar sustancialmente sus propiedades organolépticas (Patente P99 02730-5 titulada *''Procedimiento de impregnación de alimentos naturales con productos nutracéuticos y dispositivo para su puesta en práctica''*) (Betoret et al., 2003). Los gradientes de presión creados en este sistema y la presión capilar a la entrada de los poros producen una importante transferencia de gas y líquido entre los sólidos y el líquido de impregnación (Fito et al., 2001; Betoret et al., 2003; Betoret et al., 2011). Tras la impregnación a vacío, las frutas y hortalizas son muy inestables debido a su alto contenido de agua, por lo que es necesario aplicar un método de conservación para aumentar su vida útil.

Con base en la estructura porosa de algunos alimentos y la existencia de gas ocluido en su interior (Fito, 1991) explicaron el mecanismo hidrodinámico como el principal fenómeno involucrado en la operación de IV. Cuando el producto sólido, sumergido en un líquido, se somete a presiones subatmosféricas, el gas ocluido en el sólido experimenta una expansión hasta equilibrarse con las presiones alcanzadas (Betoret et al., 2015). Esto implica, por un lado, una desgasificación de la estructura porosa en función de la presión aplicada y, por otro lado, una penetración de líquido por capilaridad cuando se alcanza el equilibrio. Además, el restablecimiento de las presiones atmosféricas en el sistema promoverá un nuevo gradiente de presión que actuará como fuerza impulsora, y los espacios intercelulares del producto sólido se llenarán parcialmente con el líquido externo. La cantidad de líquido que impregne la estructura sólida dependerá del nivel de desgasificación y, por tanto, de la presión aplicada (Betoret et al., 2015).

Se puede añadir que la estructura en la que se incorporan los probióticos mediante la técnica de impregnación a vacío confiere cierta protección frente al procesado, almacenamiento y digestión.

I.2.3. Liofilización (LIO)

La liofilización es un proceso que involucra la congelación y la eliminación del agua congelada mediante sublimación y desorción en condiciones de vacío (Barbosa et al., 2015). El proceso consta de tres etapas: congelación, deshidratación primaria (sublimación) y deshidratación secundaria (desorción). Durante la congelación, los cristales de hielo extracelulares que se forman pueden provocar daños mecánicos sobre las bacterias y la concentración de solutos en el agua no congelada restante puede provocar daños químicos y osmóticos (Broeckx et al., 2016). Durante el secado, la integridad bacteriana se ve afectada ya que la eliminación de agua de las células impacta directamente en la estructura de las proteínas sensibles, la pared celular y el estado físico de las membranas lipídicas (Gómez-Zavaglia et al., 2003). Estas alteraciones de las estructuras bacterianas, que también dependen de la cepa, conducen a una disminución de su actividad metabólica, lo que a su vez conduce a una disminución de la viabilidad. Está bien documentado que la liofilización es un proceso más costoso que el secado por aspersión (alrededor de seis veces por kg de agua eliminada) y requiere mucho tiempo (Cassani et al., 2020).

Sobre la operación de liofilización se ha demostrado que la membrana celular es el elemento que más daño sufre durante la congelación y la liofilización (Gardiner et al., 2000). La lesión provocada por la congelación suele destruir la barrera de permeación iónica y, en consecuencia, se produce un colapso del potencial de membrana. Una de las razones de este daño es que las propiedades fisicoquímicas de la bicapa, como el empaquetamiento, la viscoelasticidad y la integridad de la membrana, se ven afectadas drásticamente por el cambio de temperatura y por los cambios en la relación agua/lípidos (transiciones termotrópicas y liotrópicas) (Gómez-Zavaglia et al., 2003). Estas transiciones y su implicación en las propiedades de barrera de la membrana se han estudiado ampliamente en membranas modelo de fosfatidilcolinas o fosfatidiletanolaminas que muestran una fuerte dependencia de la relación instauración/saturación de las cadenas de ácidos grasos y de los grupos polares de cabeza (Cassani et al., 2020). Sin embargo, la liofilización es la técnica más empleada para la estabilización de cepas microbianas porque, en comparación con otras técnicas de deshidratación, produce menos daños.

I.2.4. Secado por Aire Caliente (SAC)

El secado por aire caliente es el método de deshidratación más utilizado y consiste en poner en contacto una corriente de aire caliente y seco con el alimento que se quiere deshidratar. Aunque consume bastante energía, su principal ventaja es que permite reducir la humedad de los alimentos hasta un nivel seguro para evitar la multiplicación microbiana e inactivar la actividad enzimática durante el almacenamiento y transporte (Betoret et al., 2015) a menor coste que la liofilización. Preservar la calidad de los alimentos con estructura celular deshidratados por aire caliente requiere conocer cómo se transforman las estructuras durante la operación (Aguilera et al., 2003).

Para obtener productos de calidad es muy importante programar las condiciones del proceso y supervisarlo mientras se lleva a cabo, por ejemplo, controlar la actividad del agua de los alimentos que se están secando. Por lo tanto, las operaciones de secado deben controlarse y optimizarse con precisión para producir un producto de buena calidad con el más alto nivel de retención de nutrientes y sabor junto con la seguridad microbiana (Betoret et al., 2015). La textura es una de las características más afectadas y está condicionada por el efecto de las condiciones del secado en la estructura de los alimentos. Los materiales conservados por deshidratación varían mucho, desde frutas y verduras hasta microorganismos probióticos y productos de origen animal.

Según detallan Betoret et al. (2015), las altas temperaturas empleadas en la mayoría de los procesos de secado por aire caliente (entre 45 y 80 °C) y de secado por aspersión (entre 125 y 140 °C) causan daños irreversibles debido principalmente a lo siguiente:

- Cambios en las estructuras celulares (pared y membrana celulares) y cambios en las propiedades responsables de la funcionalidad del producto (la permeabilidad de la membrana celular, la resistencia mecánica del conjunto pared-membrana, etc.).
- Cambios en las estructuras químicas responsables del valor biológico de los componentes nutricionales (por ejemplo, proteínas y grasas) y de la funcionalidad tecnológica que estos compuestos dan al alimento al que pertenecen.
- Reacciones, principalmente oxidación, que disminuyen el valor funcional de compuestos nutritivos (por ejemplo, vitaminas, antioxidantes).

En alimentos con probióticos, incluso el secado con aire a temperaturas moderadas (< 40 °C) produce un descenso notable en el contenido microbiano, que incluso se anula tras 15 días de almacenamiento en ausencia de luz y oxígeno (Cassani et al., 2020). Para prevenir esta pérdida de viabilidad y proteger a los probióticos durante los procesos de deshidratación se suelen incorporar protectores a la formulación de los alimentos, algunos de los cuales se detallan a continuación.

I.2.5. Incorporación de agentes protectores

Los carbohidratos han sido, con diferencia, los compuestos protectores más utilizados durante la deshidratación, el almacenamiento y la exposición al tracto gastrointestinal de los productos formulados con probióticos. La capacidad protectora de los azúcares se puede explicar considerando dos hipótesis principales: la vitrificación y el reemplazo de agua.

- **Vitrificación**

Los vidrios son líquidos superenfriados de muy alta viscosidad, caracterizados por una alta restricción de reacciones químicas y una severa disminución de los movimientos moleculares rotacionales y vibracionales (Romano et al., 2016). Los carbohidratos se utilizan generalmente como compuestos protectores y su capacidad para formar estados amorfos (o vítreos) se utilizó para explicar el efecto protector de la mayoría de

ellos. De esta manera, las bacterias deshidratadas permanecen incrustadas en la matriz vítrea con una estabilidad química y física mejorada (Broeckx et al., 2016).

- **Reposición de agua**

La hipótesis del reemplazo de agua establece que los carbohidratos pueden reemplazar las moléculas de agua al establecer enlaces de hidrógeno con las cabezas de lípidos polares. En estado anhidro, los lípidos están muy compactos debido a la pérdida del agua de hidratación de su cabeza polar. Esto promueve la transición de las membranas lipídicas, de la fase cristalina líquida a la fase de gel (Cassani et al., 2020). A su vez, aumentan las interacciones de van der Waals, que conduce a lípidos más compactos y membranas más permeables. Hidratos de carbono, en particular los de bajo peso molecular (mono, di y trisacáridos), pueden interactuar con los grupos polares (Cassani et al., 2020) y reemplazar las moléculas de agua. Esto conduce a una disminución de la temperatura de transición de fase, estabilizando las membranas lipídicas que pueden permanecer en estado líquido cristalino en presencia de azúcares. En este contexto, el mecanismo propuesto para la acción protectora de la trehalosa es su capacidad de reemplazar el agua en la región del grupo de la cabeza polar y en las estructuras proteicas (Leslie et al., 1995).

Otros agentes protectores comúnmente utilizados para mejorar la viabilidad de los probióticos durante el procesado, el almacenamiento y el tránsito a través del tracto gastrointestinal incluyen proteínas (de origen lácteo), hidrocoloides (gelatina, goma arábiga) y polialcoholes (Cassani et al., 2020). Otros carbohidratos también han demostrado tener un papel protector. Este es el caso de los compuestos prebióticos y la fibra (la mayoría son carbohidratos), que recientemente han sido reportados como compuestos protectores adecuados (Cejas et al., 2017; Romano et al., 2016 y 2018).

I.2.6. Microencapsulación

Una estrategia muy útil para la supervivencia de bacterias probióticas es la microencapsulación. De esta forma se pueden mantener tasas de supervivencia y viabilidad más altas durante el procesado, durante el almacenamiento y después del consumo, en comparación con las células no encapsuladas (Betoret et al., 2019). Esta tecnología se define como el envasado de materiales sólidos, líquidos o gaseosos en minicápsulas que liberan su contenido en cantidades controladas durante un período prolongado. Esta tecnología encuentra un nuevo lugar en la industria alimentaria en los últimos años, además de su uso en el sector farmacéutico (Guldiken et al., 2021).

Entre las técnicas empleadas para la encapsulación de componentes bioactivos se encuentra la aplicación de altas presiones de homogeneización (HPH) que produce fuerzas disruptivas intensas que rompen las partículas en partículas más pequeñas, favoreciendo la encapsulación de componentes específicos en un medio adecuado (Mesa et al., 2020).

En algunos estudios recientes, la técnica HPH se ha empleado en la microencapsulación de células probióticas viables. Patrignani et al. (2017) constataron el potencial de la

técnica HPH para la microencapsulación realizado a 50 MPa durante cinco ciclos, utilizando alginato de sodio en emulsión con aceite vegetal, permitió obtener un rendimiento de encapsulación de *Lactobacillus paracasei* A13 y *Lactobacillus salivarius* CECT 4063 viables del 87% y 83%, respectivamente. Sin embargo, el estudio carecía de una investigación profunda sobre la dinámica de la liberación de bacterias probióticas después de la ingestión. Betoret et al. (2019), cubrieron esta brecha de conocimiento al investigar la supervivencia de *Lactobacillus salivarius* spp. *salivarius* CECT 4063 encapsulado en zumo de clementina mediante altas presiones de homogeneización (dos pasadas a 70 MPa) e incorporado en láminas de manzana mediante impregnación a vacío tras la digestión *in vitro*. Los resultados demostraron que el microorganismo encapsulado presentó una mayor resistencia a la simulación gastrointestinal en comparación con su forma libre, pero que la eficiencia de la técnica de encapsulación sobre la supervivencia del mismo durante la digestión *in vitro* dependía del tiempo de almacenamiento (Calabuig-Jiménez et al., 2019).

A continuación, se describen otras técnicas habitualmente empleadas en la producción de microcápsulas que contienen probióticos.

a) Extrusión

Es la técnica más empleada debido a su simplicidad, bajo coste y condiciones de formulación moderadas que aseguran una alta viabilidad celular (Krasaekoopt et al., 2003). Implica preparar una solución de hidrocoloide, agregar microorganismos y extruir la suspensión celular a través de una aguja de jeringa. La suspensión se gotea en una solución de endurecimiento (Heidebach et al., 2012). Si la formación de gotas se produce de forma controlada (al contrario de la pulverización), la técnica se conoce como prilling. Esto se hace por pulsación del chorro o vibración de la boquilla. El uso de flujo coaxial o un campo electrostático es la otra técnica común para formar pequeñas gotas. Cuando se aplica un campo electrostático, las fuerzas electrostáticas interrumpen el líquido superficial en la punta de la aguja, formando una corriente cargada de pequeñas gotas.

b) Emulsificación

En esta técnica, la fase discontinua (suspensión de polímero celular) se agrega a un gran volumen de aceite (fase continua). La mezcla se homogeneiza para formar una emulsión de agua en aceite. Una vez que se forma la emulsión, el polímero soluble en agua se insolubiliza (entrecruza) para formar las partículas dentro de la fase oleosa (Heidebach et al., 2012), perlas se recogen más tarde por filtración. Las medidas de las perlas están controladas por la velocidad de agitación y pueden variar entre 25 µm y 2 mm. Para aplicaciones alimentarias, se utilizan como fase oleosa los aceites vegetales. Algunos estudios han utilizado aceite de parafina de luz blanca y aceite mineral. También se agregan emulsionantes para formar una mejor emulsión porque los emulsionantes reducen la tensión superficial, lo que da como resultado partículas más pequeñas (Rodriguez-Concepcion et al., 2018).

c) Secado por aspersión

Es un método rápido, continuo y rentable, y permite la deshidratación de grandes cantidades de cultivos de alimentos líquidos en un tiempo relativamente corto y a gran escala. En este proceso las suspensiones bacterianas se atomizan en gotas en una cámara de secado donde un flujo controlado de aire caliente a temperaturas de hasta 200 °C evapora rápidamente las pequeñas gotas. En la industria alimentaria está técnica es utilizada en la fabricación de la leche en polvo, pero los atomizadores se podrían adaptar a otros fines también (Cassani et al., 2020). A pesar de estas ventajas, los microorganismos están expuestos a varios estreses durante el proceso, incluyendo estrés térmico, deshidratación, cizallamiento, estrés osmótico y oxidativo, que resultan en la pérdida de viabilidad. La tolerancia a las tensiones mencionadas depende de la especie y la cepa. Por ejemplo, la mayoría de las cepas de propionibacterias han mostrado una mayor tolerancia que las de lactobacilos y bifidobacterias. También las cepas de estreptococos suelen soportar mejor el proceso de secado por aspersión que los lactobacilos debido a su alta termotolerancia (Huang et al., 2017).

Los iniciadores de probióticos se suministran con frecuencia en formatos congelados o secos (es decir, polvos encapsulados, liofilizados o atomizados). El manejo de cultivos congelados tiene algunas desventajas, como el requisito de temperaturas de almacenamiento bajo cero, lo que no solo causa lesiones celulares en las estructuras bacterianas como resultado de los procesos de congelación y descongelación, sino que también implica altos costos de energía y transporte (Cassani et al., 2020).

I.3. Nutrición personalizada a través del estudio del microbiota. Nutrigenómica y el futuro de los nutraceúticos

La vida es un fenómeno colectivo. Cada uno de nosotros convive directamente con un número muy alto de microorganismos (bacterias, protozoos, levaduras y virus) que están alojados en nuestro cuerpo, muchos de ellos de forma permanente. El microbioma humano se define como el conjunto de microbios, sus genes y productos que colonizan nuestro cuerpo desde el nacimiento.

En cambio, la microbiota intestinal hace referencia a la comunidad de microorganismos vivos reunidos en el intestino y que desempeña un papel vital en el mantenimiento de la homeostasis intestinal, la regulación de la inmunidad y metabolismo del huésped (Kumari y Kokkiligadda, 2021). La microbiota está compuesta principalmente por bacterias estrictamente anaeróbicas, que se han adaptado a la vida en las superficies mucosas o en la luz del intestino desde hace milenios (Suissa et al., 2022). Pero, además, ese colectivo de seres vivientes aporta un conjunto de genes, sus genes, que también intervienen en muchos de nuestros procesos biológicos (Vos et al., 2022) . El conjunto de genes distintos de los nuestros que influye continuamente en nuestra vida es lo que llamamos metagenoma humano.

Varios factores pueden afectar la composición de la microbiota intestinal a lo largo de la vida, como por ejemplo la genética del huésped, la edad, el sexo, la dieta, los factores ambientales y las terapias, especialmente los antibióticos. Estos factores pueden ayudar a determinar la influencia de la microbiota intestinal en la salud y las enfermedades humanas contribuyendo a la diversidad y singularidad de la microbiota intestinal en cada individuo (Hasan y Yang, 2019).

Con los avances en las técnicas moleculares, la investigación ha demostrado que la microbiota del intestino humano adulto incluye unos 100 billones de bacterias de unas 500 a 1000 especies distintas; de modo que el número de células bacterianas es 10 veces más grande que el número de células somáticas, siendo las especies gram positivas anaeróbicas las que se encuentran con mayor frecuencia (Álvarez Calatayud et al., 2018).

Aunque las especies varían significativamente de una persona a otra, los principales filos bacterianos intestinales que se encuentran en la composición de la microbiota de un individuo sano son: *Firmicutes* y *Bacteroidetes,* seguidos de *Proteobacteria, Actinobacteria y Verrucomicrobia*. El filo *Firmicutes* consta principalmente de miembros de los géneros *Clostridium, Lactobacillus* y *Ruminococcus*, así como de los productores de butirato *Eubacterium*, *Fecalibacterium* y *Roseburia*. El filo *Bacteroidetes* consta de géneros predominantes de *Bacteroides, Prevotella y Xylanibacter* que son reconocidos por su capacidad para degradar las fibras dietéticas. El filo *Actinobacteria* está representado principalmente por el género *Bifidobacterium*. El filo *Proteobacteria* incluye el género *Escherichia* y *Desulfovibrio*, mientras que el filo *Verrucomicrobia* hasta ahora comprende solo el género *Akkermansia* (Kumari y Kokkiligadda, 2021).

Los resultados basados en datos experimentales han identificado tres funciones principales de la microbiota, identificándola como un órgano metabólico que realiza múltiples funciones importantes para la nutrición y la salud de su huésped (Guarner y Malagelada, 2003).

a) Funciones de nutrición y metabolismo, como resultado de la actividad bioquímica de la flora, que incluyen recuperación de energía en forma de ácidos grasos de cadena corta, producción de vitaminas y efectos favorables sobre la absorción de calcio y hierro en el colon. Las funciones metabólicas también incluyen la producción de vitaminas (K, B12, biotina, ácido fólico y pantoténico) y la síntesis de aminoácidos a partir del amoniaco o la urea (Hepatología et al., 2009).

b) Funciones de protección, previos a la invasión de agentes infecciosos o el sobrecrecimiento de especies residentes con potencial patógeno.

c) Funciones tróficas sobre la proliferación y diferenciación del epitelio intestinal, y sobre el desarrollo y modulación del sistema inmune.

Las investigaciones llevadas a cabo en el campo de la interacción huésped-microbioma han permitido comprender los conceptos de colonización del microbiota y su respuesta mediada a varias enfermedades en el huésped. Esta observación ha promovido el

desarrollo de estrategias terapéuticas dirigidas al microbioma en enfermedades relacionadas con la disbiosis, incluida la administración de probióticos, prebióticos y sinbióticos o mediante la repoblación con especies bacterianas comensales a través de una infusión de trasplante de microbiota fecal (FMT) (Kumari y Kokkiligadda, 2021).

Diversos estudios respaldan el hecho de que los probióticos son útiles para la erradicación de la resistencia a los antibióticos mediante trasplante fecal para descolonizar cepas bacterianas naturalmente resistentes (Chaudhari y Dwivedi, 2022). Es el caso de la infección recurrente por *Clostridium difficile* – Recurrent *Clostridium difficile* Infection (RCDI) por sus siglas en inglés, que se asocia con el tratamiento repetido con antibióticos y el aumento del crecimiento de microbios resistentes a los antibióticos. (Millan et al., 2016), probaron que los pacientes con RCDI albergarían una gran cantidad de microbios resistentes a los antibióticos y que el trasplante de microbiota fecal (FMT) reduciría la cantidad de genes resistentes a los antibióticos. Sin embargo, aunque FMT es eficaz en el tratamiento de la infección recurrente por *Clostridium difficile*, su uso en otras enfermedades metabólicas sigue siendo difícil de alcanzar (Kumari y Kokkiligadda, 2021).

El conocimiento de la composición de la microbiota intestinal humana y su relación con determinadas enfermedades ha crecido enormemente gracias al avance reciente en las técnicas moleculares. Los probióticos pueden ayudar a mantener la homeostasis dentro del TGI y pueden ser utilizados como productos bioterapéuticos vivos, dando lugar a una nueva clase de probióticos denominados probióticos de nueva generación Next Generation Probiotics (NGP) por sus siglas en inglés (Zhang et al., 2019).

Los NGP se ajustan por una parte a la definición del probiótico de la FAO/OMS (Organización para la Agricultura y la Alimentación/Organización Mundial de la Salud) y, por otra parte, cumplen los criterios incluidos en la definición de los productos bioterapéuticos vivos - live biotherapeutics products (LBP), por parte de la Administración de Alimentos y Medicamentos (FDA) de los Estados Unidos: “un producto biológico que contiene organismos vivos; es aplicable a la prevención, tratamiento o cura de una enfermedad o condición de los seres humanos; y que no es una vacuna” (Kumar y Kokkiligadda, 2021). Los NGP se pueden obtener a partir de las bacterias comensales que están vinculadas a la homeostasis intestinal y la salud humana. Algunas especies interesantes para tal fin incluyen cepas de *Akkermansia muciniphila, Bacteroides fragilis, Faecalibacterium prausnitzii, Eubacterium hallii y Parabacteroides goldsteinii.*

Akkermansia muciniphila

Esta especie de bacteria es abundante en el tracto intestinal humano y representa del 0,01 % a casi el 4 % de la carga bacteriana total del intestino. Además del colon, *A. muciniphila* se encuentra en la leche humana, la cavidad oral, el páncreas, el sistema biliar, el intestino delgado y el apéndice (Geerlings et al., 2018).

A. muciniphila es capaz de sintetizar todos los aminoácidos esenciales, excepto la L-treonina, que se encuentra entre los aminoácidos más frecuentes en la estructura

proteica de la mucina (Ottman et al., 2017). También se ha relacionado con diversas enfermedades metabólicas como Síndrome del Intestino Irritable - Irritable Bowel Syndrome por sus siglas en inglés (IBS), obesidad, diabetes tipo 2 y autismo. *Akkermansia muciniphila* es un anaerobio intestinal que ha sido propuesto como un nuevo microbio funcional con propiedades probióticas. Sin embargo, la especie no está de momento incluida en la lista de presunción de seguridad calificada - Qualified Presumption of Safety (QPS) de la EFSA.

Bacteroides fragilis

B. fragilis no toxígena (Non-Toxigenic *B. fragilis* - NTBF), es un comensal beneficioso del intestino humano, que puede utilizarse como NGP emergente para mejorar las enfermedades relacionadas con la inflamación, incluidas las enfermedades autoinmunes. Esta bacteria se encuentra comúnmente en la superficie mucosa del tracto gastrointestinal, pero también se ha identificado en la boca, el tracto respiratorio superior y el tracto genital femenino.

Los efectos que ejerce *B. fragilis* sobre la salud están relacionados con la erradicación de varios patógenos y su infección asociada, incluidos *Helicobacter hepaticus, Clostridium perfringens*, C. *difficile* entre otros (Kumari y Kokkiligadda, 2021). *B. fragilis* y su polisacárido A tienen efectos significativos en la prevención y tratamiento de enfermedades relacionadas con el intestino como colitis, cáncer colorrectal, trastornos neurológicos y esclerosis múltiple, estimulando la producción de citocinas antiinflamatorias, fortaleciendo la integridad de la barrera intestinal y modulando la composición microbiana intestinal (Kumari y Kokkiligadda, 2021).

En relación con los aspectos de seguridad de esta bacteria, algunos estudios demuestran que *B. fragilis* es seguro y genéticamente estable en ratones normales e inmunodeficientes, sin riesgo de transferir el gen de resistencia a los antibióticos. Sin embargo, la investigación de *B. fragilis* se limita a la etapa de experimentación con animales, y se requieren más estudios clínicos que permitan demostrar la seguridad de este microorganismo (Wang et al., 2017).

Faecalibacterium prausnitzii

F. prausnitzii es una de las bacterias anaerobias más numerosas en la microbiota intestinal humana y representa entre el 5% y el 15% del total de bacterias detectables en muestras fecales de sujetos sanos (Fitzgerald et al., 2018). Esta bacteria candidata a ser NGP, ha demostrado a través de numerosos estudios recientes tener efectos beneficiosos en las enfermedades intestinales y ser un potencial marcador de la salud intestinal humana.

F. prausnitzii produce butirato mediante la alimentación cruzada de acetato y propionato producido por mucina que degrada *A. muciniphila* (Belzer et al., 2017). El butirato actúa como la principal fuente de energía para las células epiteliales y desempeña un papel crucial en el mantenimiento de un intestino sano, la producción de

compuestos antiinflamatorios, la mejora de la integridad de la barrera intestinal y la modulación del estrés oxidativo y de la carcinogénesis (Kumari y Kokkiligadda, 2021).

La evaluación de la seguridad de *F. prausnitzii* se limita a unos pocos estudios. Se necesitan más investigaciones que confirmen las características de esta bacteria, como la resistencia natural o de tipo adquirido a los antibióticos, y otros parámetros de seguridad.

Eubacterium hallii

E. hallii es una bacteria grampositiva, estrictamente anaeróbica, que representa alrededor del 2% - 3% del total de bacterias fecales presentes en individuos sanos. Es una bacteria intestinal productora de butirato (Louis y Flint, 2009). El crecimiento de *E. hallii* y la producción de butirato pueden ser estimulados por la presencia de productos finales metabólicos como lactato y acetato producidos por bacterias *Lactobacilos* y *Bifidobacterias* a partir de fructanos de tipo inulina. Por esta razón, *E. hallii* está ganando popularidad debido a su potencial para influir en la homeostasis del huésped, así como en la microbiota intestinal a través de sus metabolitos (Kumari y Kokkiligadda, 2021).

Udayappan et al. (2016) demostraron que la administración oral de la cepa *E. hallii*, incluso en dosis altas, no causó ningún efecto adverso en ratones obesos y diabéticos en comparación con el grupo de control sano. Este resultado respalda la evidencia de seguridad y potencial emergente de *E. Hallii* como candidato a probiótico de nueva generación.

Parabacteroides goldsteinii

P. goldsteinii es un comensal que podría ser un nuevo candidato probiótico de nueva generación para tratar la obesidad. Es una bacteria anaeróbica obligada, que no forma esporas, no es móvil, es una bacteria gram negativa y que se aísla de la sangre humana.

En relación con los efectos sobre la salud de *P. goldsteinii*, los resultados de diferentes investigaciones llevadas a cabo en animales muestran que este probiótico se puede usar para tratar enfermedades metabólicas como la diabetes tipo 2, la obesidad y la enfermedad del hígado graso, al reducir la resistencia a la insulina y mejorar la tolerancia a la glucosa disminuyendo el peso corporal, la acumulación de grasa, el tamaño de los adipocitos, el peso del hígado, la acumulación de lípidos hepáticos y la hipertrofia de hepatocitos en modelos animales (Kumari y Kokkiligadda, 2021).

Teniendo en cuenta que hay pocos estudios clínicos en humanos y para evitar posibles problemas de seguridad se necesita más investigación que ayude a comprender los efectos beneficiosos reales o los efectos nocivos potenciales de *P. goldsteinii* antes de usarla como tratamiento para enfermedades metabólicas como la obesidad.

En términos generales puede afirmarse que la diferencia en términos de su patogenicidad potencial entre las especies probióticas tradicionales de *Lactobacillus* y *Bifidobacterium* y los NGP es un punto clave. Esto hace que la evaluación de la seguridad sea la preocupación más obvia para identificar a candidatos potenciales a NGP. Los

factores que hay que considerar en esta evaluación son la clasificación taxonómica distintiva a nivel de especie, el perfil completo de la cepa para la identificación de toxinas y el gen relacionado con la virulencia, la resistencia a los antibióticos y la posible transferencia horizontal, y otras actividades metabólicas adversas en general (Saarela, 2019; Kumari y Kokkiligadda, 2021).

La nutrición es un factor clave y permanente en el desarrollo de todos los seres vivos, que implica la interacción entre los genes y los componentes bioactivos de la dieta, y la influencia de otros factores como el estado socioeconómico, el nivel de sedentarismo/actividad, la presencia de contaminantes ambientales, entre otros. Esta interacción al mismo tiempo está condicionada por las características genéticas del individuo (Gottlieb et al., 2020). La nutrigenómica y la nutrigenética son ramas de la biotecnología que tratan de establecer la relación entre estos factores; la nutrigenómica tiene como objetivo conocer la relación entre los componentes bioactivos ingeridos en la dieta y la expresión de los genes que regulan la respuesta fisiológica asociada (Braicu et al., 2017).

Por su parte, la nutrigenética estudia la relación entre los genes y la respuesta individual a la dieta. Dicho de otro modo, se encarga de investigar cómo las diferencias propias en los genes influencian los procesos nutricionales a corto y largo plazo. El objetivo es prevenir las patologías relacionadas con la ingesta y, en caso de aparecer, tratar de abordarlas de la manera más eficaz posible. El avance en este conocimiento dirige la nutrición hacia una “nutrición personalizada” o “nutrición individualizada” (Ordovas y Corella, 2004).

Gracias a los numerosos esfuerzos y al avance en el análisis óhmico, la comunidad científica avanza gradualmente hacia la medicina personalizada y la era del microbioma es claramente una parte importante del cambio de paradigma en el futuro de la medicina y los enfoques nutricionales (Vos et al., 2022).

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II. OBJETIVOS

II.1. Objetivo general

El objetivo general de esta tesis doctoral consiste en estudiar el efecto que la adición de trehalosa, la aplicación de altas presiones de homogeneización (HPH) y la inclusión en una matriz alimentaria ejercen sobre las propiedades antioxidantes y la resistencia de *Lactobacillus salivarius* spp. *salivarius* CECT 4063 al procesado, almacenamiento y digestión gastrointestinal *in vitro* cuando se incluye en un snack de manzana impregnado con zumo de clementina.

II.2 Objetivos específicos

Para alcanzar el objetivo general se plantearon los siguientes objetivos específicos:

1. Determinar el efecto de la trehalosa y de las altas presiones de homogenización (HPH) sobre las propiedades fisicoquímicas, incluido el contenido en compuestos con actividad antioxidante, del zumo de clementina sin inocular.

2. Evaluar el efecto de la trehalosa y de las altas presiones de homogenización (HPH) sobre las propiedades microbiológicas, incluido el crecimiento microbiano y la capacidad del microorganismo para adherirse a la mucosa intestinal, y para resistir a las condiciones del tracto gastrointestinal, del zumo de clementina inoculado y fermentado con *Lactobacillus salivarius* spp. *salivarius* CECT 4063.

3. Analizar el efecto de la fermentación del zumo de clementina con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 sobre las propiedades fisicoquímicas, incluido el contenido en compuestos con actividad antioxidante, en función de la adición de trehalosa y de la aplicación de altas presiones de homogeneización.

4. Determinar el efecto de la trehalosa y de las altas presiones de homogenización (HPH) sobre la estabilidad de las propiedades antioxidantes y microbiológicas del zumo de clementina inoculado y fermentado con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 durante el almacenamiento en refrigeración.

5. Analizar el efecto de la trehalosa y de las altas presiones de homogenización (HPH) sobre la cantidad de zumo de clementina inoculado y fermentado con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 que es posible incorporar por impregnación a vacío en la matriz estructural de láminas de manzana (var. *Granny Smith*).

6. Evaluar el efecto que la adición de trehalosa al zumo de clementina antes de la inoculación con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 y/o la homogeneización del zumo fermentado ejercen sobre la resistencia a la liofilización y al secado con aire a 40 °C durante 24 y 48 h de los compuestos antioxidantes y el microorganismo incorporado a láminas de manzana (var. *Granny Smith*) mediante la técnica de impregnación a vacío.

7. Evaluar el efecto que la adición de trehalosa al zumo de clementina antes de la inoculación con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 y/o la

homogeneización del zumo fermentado, así como el nivel de actividad del agua alcanzado por el snack de manzana tras el proceso de deshidratación, ejercen sobre la viabilidad del microorganismo y la estabilidad de las propiedades antioxidantes durante el almacenamiento.

8. Analizar el efecto que la adición de trehalosa al zumo de clementina antes de la inoculación con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 y/o la homogeneización del zumo fermentado, así como el tipo de matriz alimentaria, ejercen sobre la resistencia de *Lactobacillus salivarius* spp. *salivarius* CECT 4063 a su paso por el tracto gastrointestinal.

III. PLAN DE TRABAJO

III.1. Plan de trabajo

A continuación, se detallan las experiencias que, tras la revisión bibliográfica necesaria para recopilar la información más relevante en relación con el desarrollo de alimentos probióticos a partir de matrices vegetales y con las estrategias empleadas para aumentar la funcionalidad de estos, se propusieron para la consecución del objetivo general antes mencionado.

1. Ensayos de fermentación a 37 °C durante 24 h de zumo de mandarina comercial rectificado y formulado con diferentes concentraciones de trehalosa (0, 10 y 20 g/100 g), según se detalla en la Figura 3.
    - Recuento de viables en el zumo fermentado.
    - Análisis de las propiedades antioxidantes (contenido en vitamina C, contenido en fenoles y flavonoides totales y actividad antioxidante por los métodos ABTS-TEAC y DPPH) del zumo antes y después de la fermentación.
    - Análisis de la actividad del agua (a_w), el pH, el contenido en sólidos solubles (ºBrix) y el tamaño de partícula del zumo antes y después de la fermentación.

2. Ensayos de homogeneización a diferentes presiones (25, 50, 100 y 150 MPa) de zumo de mandarina comercial rectificado, formulado con diferentes concentraciones de trehalosa (0, 10 y 20 g/100 g) y fermentado con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 durante 24 h a 37 °C (Figura 2.1).
    - Recuento de viables antes y después de la homogeneización.
    - Análisis de las propiedades antioxidantes (contenido en vitamina C, contenido en fenoles y flavonoides totales y actividad antioxidante por los métodos ABTS-TEAC y DPPH) del zumo fermentado, homogeneizado y sin homogeneizar.
    - Análisis de la actividad del agua (a_w), el pH, el contenido en sólidos solubles (ºBrix) y el tamaño de partícula del zumo fermentado, antes y después de la homogeneización.
    - Análisis de la capacidad del zumo fermentado, homogeneizado y sin homogeneizar, para inhibir el crecimiento de *Helicobacter pylori*.
    - Análisis de la capacidad del zumo fermentado, homogeneizado y sin homogeneizar, para adherirse a las proteínas de la mucosa gástrica (colágeno, mucina y albúmina de suero bovino).

3. Ensayos de fermentación a 37 °C durante 96 h de zumo de mandarina comercial, con y sin 10 g de trehalosa /100 g, sin homogeneizar y homogeneizado a 100 MPa antes de la inoculación con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 o tras 24 h de fermentación del zumo inoculado (Figura 4).
    - Recuento de viables durante la fermentación.
    - Análisis de las propiedades antioxidantes del zumo (contenido en fenoles y flavonoides totales y actividad antioxidante por los métodos ABTS-TEAC y DPPH) durante la fermentación.
    - Análisis mediante HPLC del contenido en flavonoides específicos del zumo (narirrutina, didimina y hesperidina) durante la fermentación.

4. Ensayos de almacenamiento en refrigeración a 4 °C durante 30 días de zumo de clementina comercial, con y sin 10 g de trehalosa /100 g, fermentado durante 24 h a 37 °C con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 y homogeneizado o no a 100 MPa (Figura2.2).
    - Recuento de viables durante el almacenamiento.
    - Análisis de las propiedades antioxidantes del zumo (contenido en fenoles y flavonoides totales y actividad antioxidante por los métodos ABTS-TEAC y DPPH) durante el almacenamiento.

5. Ensayos de impregnación a vacío de láminas de manzana (var. *Granny Smith*) de 5 mm de espesor con zumo de mandarina comercial formulado con diferentes concentraciones de trehalosa (0 y 10 g/100 g), fermentado con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 durante 24 h a 37 °C y homogeneizado a diferentes presiones (0, 50 y 100 MPa).
    - Determinación de los parámetros de impregnación.
    - Análisis de las propiedades antioxidantes (contenido en fenoles y flavonoides totales y actividad antioxidante por los métodos ABTS-TEAC y DPPH) de la manzana fresca e impregnada.
    - Análisis del color, la actividad del agua (a_w), la humedad (x_w) y las propiedades mecánicas de la manzana fresca e impregnada.

6. Estudios de deshidratación mediante liofilización o secado con aire a 40 °C durante 24 y 48 h de láminas de manzana impregnadas con diferentes líquidos probióticos a base de zumo de clementina comercial y *Lactobacillus salivarius* spp. *salivarius* CECT 4063.
    - Recuento de viables antes y después de la deshidratación.
    - Análisis de las propiedades antioxidantes (contenido en fenoles y flavonoides totales y actividad antioxidante por los métodos ABTS-TEAC y DPPH) de la manzana deshidratada.
    - Análisis del color, la actividad del agua (a_w), la humedad (x_w) y las propiedades mecánicas de la manzana deshidratada (Figura 5).

7. Estudios de almacenamiento a temperatura ambiente durante 30 días de los snacks de manzana, obtenidos mediante impregnación con diferentes líquidos probióticos a base de zumo de clementina comercial y *Lactobacillus salivarius* spp. *salivarius* CECT 4063 y posterior deshidratación mediante liofilización o secado con aire a 40 °C durante 24 y 48 h.
    - Recuento de viables durante el almacenamiento.
    - Análisis de las propiedades antioxidantes (contenido en fenoles y flavonoides totales y actividad antioxidante por los métodos ABTS-TEAC y DPPH) durante el almacenamiento.

8. Ensayos de digestión *in vitro* para evaluar el efecto de la presencia de trehalosa y de la aplicación de altas presiones de homogeneización sobre la bioaccesibilidad del *Lactobacillus salivarius* spp. *salivarius* CECT 4063 y de los

compuestos antioxidantes presentes en diferentes matrices alimentarias (zumo de clementina, láminas de manzana impregnadas y snacks de manzana obtenidos mediante impregnación a vacío y liofilización o secado con aire a 40 °C durante 48 h).

- Recuento de viables durante el proceso de digestión *in vitro*, análisis de las propiedades antioxidantes (contenido en fenoles y flavonoides totales y actividad antioxidante por los métodos ABTS-TEAC y DPPH) durante la simulación gastrointestinal.

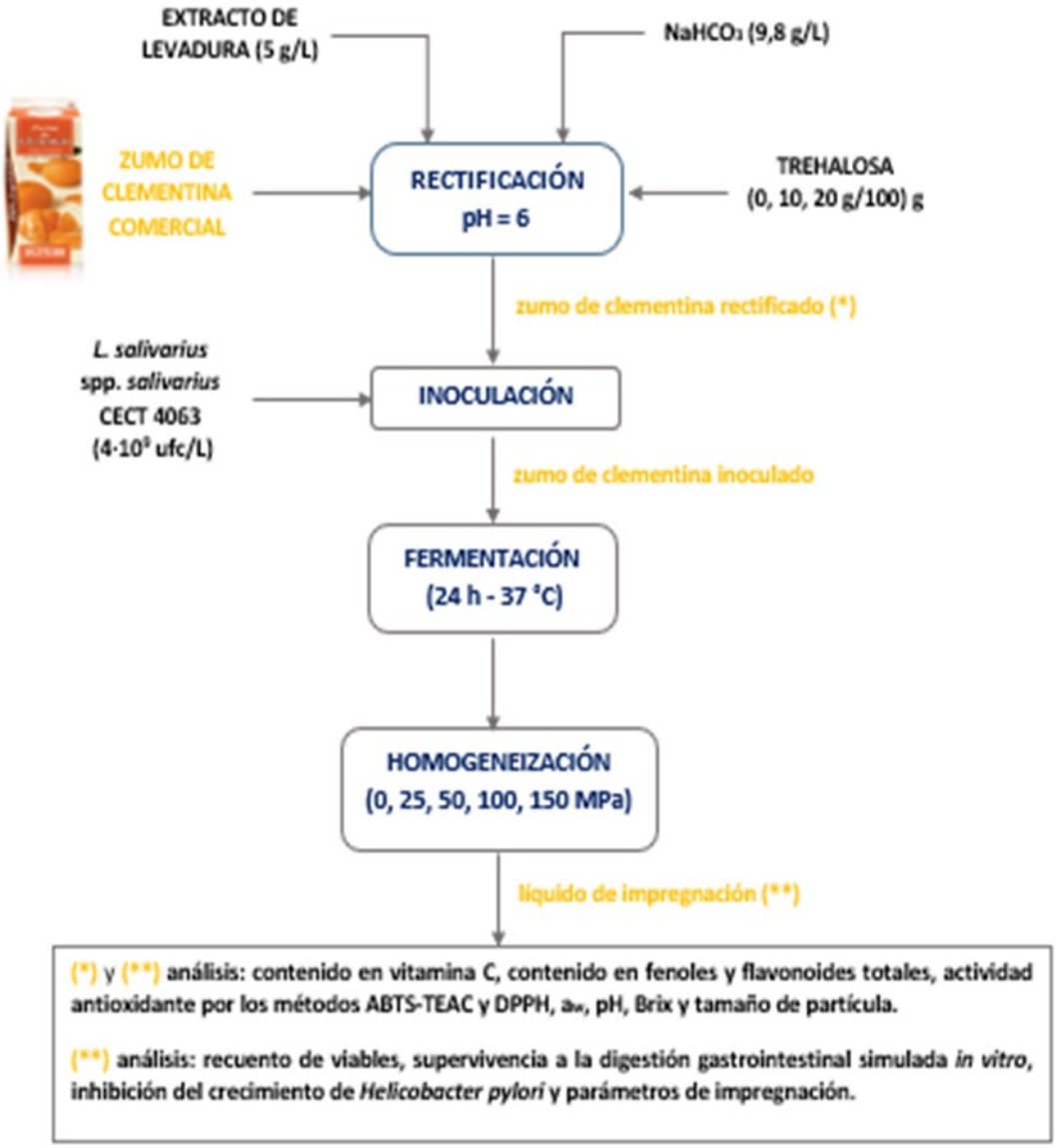

Figura 3. Diagrama de flujo que describe el proceso seguido para evaluar el efecto del tratamiento con altas presiones de homogeneización (HPH) y/o la adición de trehalosa sobre las propiedades tecnológicas y funcionales del zumo de clementina fermentado y sin fermentar con *L. salivarius* spp. *salivarius* CECT 4063.

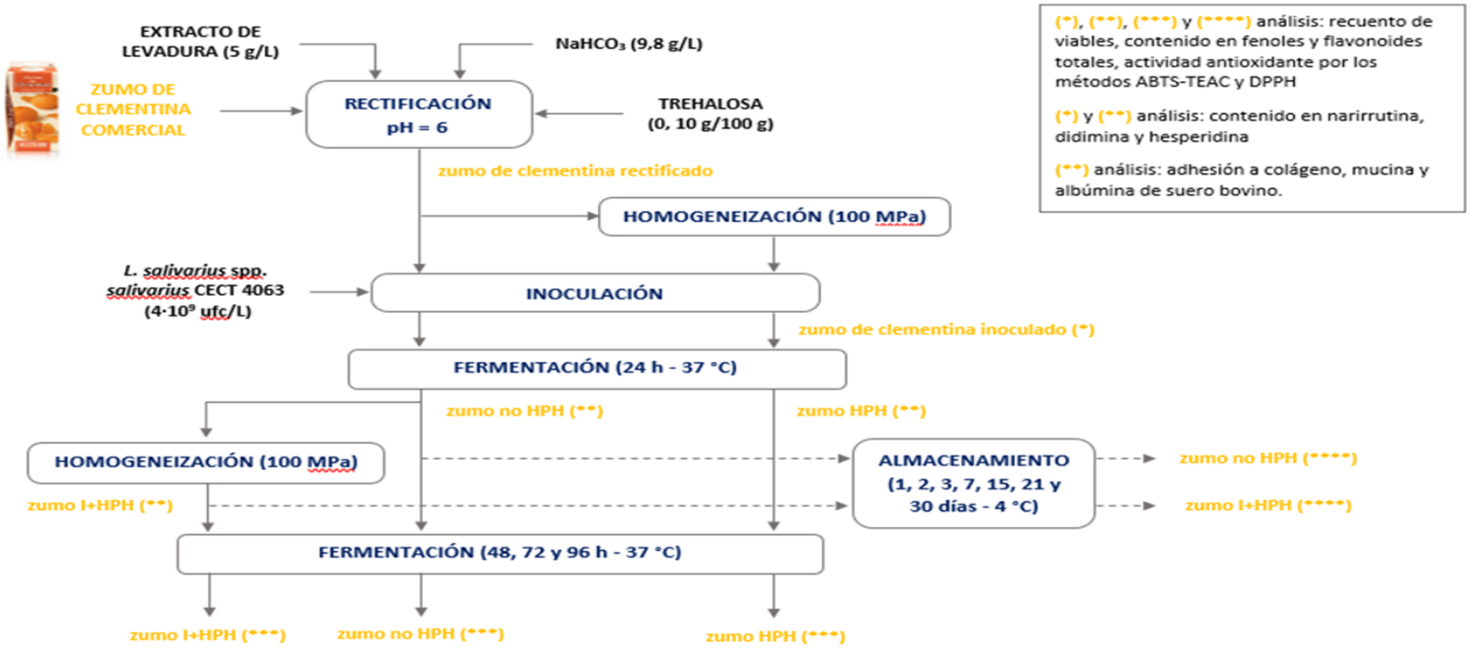

Figura 4. Diagrama de flujo que describe los estudios de fermentación a 37 °C durante 96 h de zumo de clementina comercial y de almacenamiento a 4 °C durante 30 días del zumo de clementina fermentado con *L. salivarius* spp. *salivarius* CECT 4063 en función del tratamiento con altas presiones de homogeneización (HPH) y/o la adición de trehalosa.

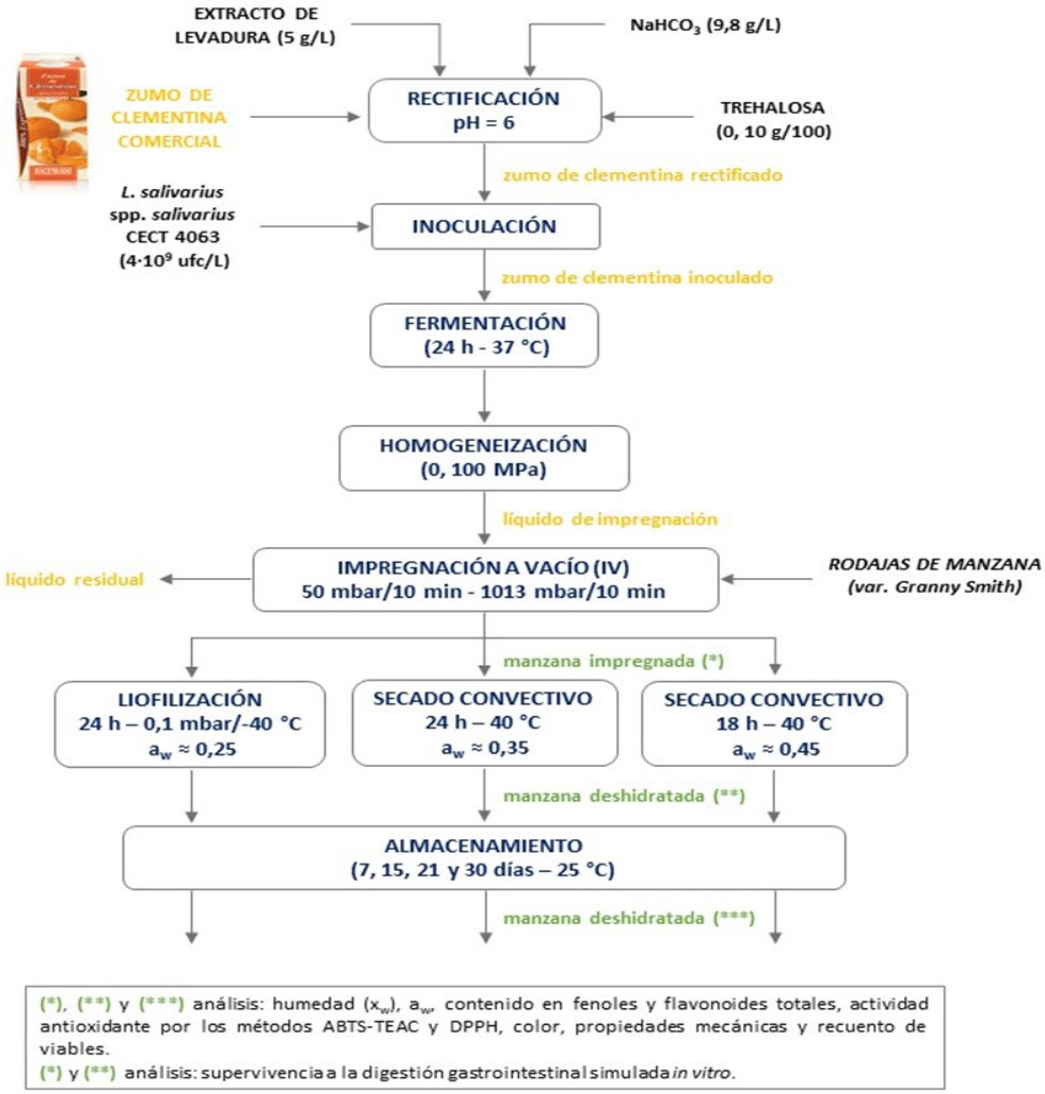

Figura 5. Diagrama de flujo seguido en el proceso de obtención del snack de manzana y la evaluación del efecto de la presión de homogeneización (HPH), la adición de trehalosa y la técnica de deshidratación sobre las propiedades fisicoquímicas y microbiológicas y su evolución durante el almacenamiento.

IV. RESULTADOS

IV. CAPÍTULO 1

IV.1 Obtención de un líquido de impregnación a base de zumo de clementina con elevado contenido en unidades formadoras de colonias de *L. salivarius* CECT 4063 que sean resistentes al almacenamiento y a la digestión simulada *in vitro*

ARTÍCULO 1

Cristina Barrera, **Cristina Burca**, Ester Betoret, Jorge García Hernández, Manuel Hernández and Noelia Betoret (2019). Improving antioxidant properties and probiotic effect of clementine juice inoculated with *Lactobacillus salivarius* spp. *salivarius* (CECT 4063) by trehalose addition and/or sublethal homogenization. *International Journal of Food Science and Technology*,54(6),2109-2122. DOI:10.1111/ijfs.14116. https://doi.org/10.1111/ijfs.14116

ARTÍCULO 2

Cristina Burca-Busaga, Noelia Betoret, Lucía Seguí and Cristina Barrera (2022). Fermentation of clementine juice with *Lactobacillus salivarius* spp. *salivarius* CECT 4063: effect of trehalose addition and high pressure homogenization on antioxidant properties, mucin adhesion and shelf-life. Fermentation Journal. *Fermentation* 2022, 8, 642.https://doi.org/10.3390/fermentation8110642. https://doi.org/10.3390/fermentation8110642

Uno de los más importantes requisitos de una cepa para poder ser considerada probiótica es que debe mantenerse viable desde la producción hasta el consumo y durante el TGI. De acuerdo con esto, la resistencia a los ácidos por parte de los probióticos es fundamental con respecto a las aplicaciones alimentarias, por ser la acidez una de las principales causas de la pérdida de viabilidad que se observa en los alimentos fermentados. De la resistencia microbiana a los ácidos depende el tiempo de permanencia en el TGI y, con ello, la posibilidad de ejercer su efecto beneficioso. La capacidad de una cepa para adherirse al moco intestinal también está relacionada con su tiempo de residencia intestinal y es un requisito previo para la colonización temporal de las superficies mucosas. Otros mecanismos por los que las cepas probióticas ejercen un efecto beneficioso para la salud del huésped son la inhibición de patógenos y la inmunomodulación.

En cuanto a los beneficios que aportan los probióticos, se pueden clasificar en nutricionales y terapéuticos. Entre los factores nutricionales se encuentra su papel para aumentar la biodisponibilidad de minerales tales como calcio, zinc, hierro, manganeso, cobre y fosforo. A nivel terapéutico se pueden utilizar para el tratamiento de desórdenes intestinales, hipercolesterolemia, para la supresión de enzimas procarcinogénicas o para la inmunomodulación, entre otros. La capacidad de las bacterias para sobrevivir durante su paso a través del tracto gastrointestinal a pesar de la acidez gástrica y la toxicidad de la bilis es fundamental para poder ofrecer cualquiera de los beneficios anteriormente mencionados.

Mediante diversos estudios científicos se ha demostrado el efecto beneficioso que ejercen las matrices alimentarias sobre el crecimiento y la supervivencia de determinados microorganismos durante el tránsito gástrico, condicionando así la composición de la microbiota humana y haciendo más recomendable el consumo de probióticos como parte de un alimento funcional que en forma de suplementos. Para conseguir alimentos probioticos con crecimientos adecuados, no solo es necesario seleccionar una cepa que se adecúe bien a esa matriz alimentaria, sino también establecer las condiciones óptimas de procesado y conservación.

En los últimos años ha aumentado el interés por desarrollar productos probióticos a partir de matrices no lácteas para responder a las necesidades nutricionales de los consumidores vegetarianos, con intolerancia a lactosa y con dietas restrictivas por el elevado nivel de colesterol. En concreto, las frutas y verduras están siendo utilizadas con estos fines y, aunque los zumos son las matrices con mejores índices de crecimiento en el procesado y en la conservación, su inclusión en la matriz estructural de diferentes vegetales mediante la técnica de impregnación a vacío permite, además, desarrollar alimentos probióticos estructurados y que conserven su integridad celular.

Entre los factores que afectan la viabilidad de los probióticos se encuentran factores relacionados con la propia composición del alimento (pH, oxígeno, presencia de sales, azúcares, etc.), factores relacionados con las condiciones de procesado y almacenamiento y factores de tipo microbiológico (cantidad inoculada, tipo de cepa, etc.). En este sentido se puede decir que, debido a los avances tecnológicos, se ha

logrado mejorar la vida útil del probiótico desde su producción, almacenamiento, hasta el momento de ser consumido. Entre las técnicas que se aplican para aumentar la resistencia de los probióticos a las condiciones adversas de procesado de alimentos destacan la adición de agentes protectores, como la trehalosa, la aplicación de altas presiones de homogeneización (HPH) y la encapsulación.

La trehalosa es un agente protector utilizado en las investigaciones recientes. Desde el punto de vista químico, representa un disacárido no reductor constituido por dos moléculas de glucosa que se encuentra en diversos organismos, en los cuales actúa como azúcar de reserva y como protector ante el estrés abiótico, al mismo tiempo que es un metabolito natural muy importante y un posible aditivo alimentario. Se sabe que este azúcar protege las membranas celulares y las proteínas contra el estrés abiótico causado por el calor, la exposición al etanol o la alta concentración de solutos en los procesos de deshidratación. Entre sus funciones, la trehalosa es capaz de mantener y preservar un extenso grupo de moléculas biológicamente activas. Este efecto es debido al establecimiento de interacciones que contribuyen a crear una barrera capaz de mantener la integridad de las estructuras celulares y prevenir su descomposición durante las operaciones de procesado y almacenamiento.

La homogeneización a alta presión (*High Pressure Homogenization* o HPH) es otra técnica que permite mejorar la funcionalidad de los probióticos. Se trata de una tecnología no térmica que se aplica para inactivar enzimas, mejorar la calidad sensorial y tecnológica de los alimentos y aumentar la disponibilidad de compuestos activos. Por otra parte, el empleo de presiones moderadas produce una mejora de las propiedades funcionales de los probióticos, tales como la hidrofobicidad, la autoagregación y la resistencia al estrés biológico. Por definición, la hidrofobicidad celular se ha relacionado con la capacidad de adhesión de la cepa y, tal y como se ha comentado anteriormente, se considera un factor importante para la interacción de bacterias probióticas con el intestino y las células inmunes asociadas a él. Se ha visto en estudios previos que la aplicación de altas presiones de homogeneización también puede incrementar la resistencia de los microorganismos probióticos a la digestión gastrointestinal. Debido a las temperaturas moderadas de trabajo, esta tecnología permite minimizar las pérdidas por calidad, producir alimentos seguros, mantener las propiedades nutricionales y sensoriales, por lo que resulta una alternativa interesante a la estabilización por medio de tratamientos térmicos.

Con respecto a las matrices alimentarias, se puede decir que las frutas y hortalizas ya de por sí son ricas en compuestos bioactivos, pero sus propiedades funcionales pueden verse mejoradas tras la fermentación con microorganismos probióticos. Según diversos estudios, el aumento en el contenido en compuestos bioactivos asociado al proceso de fermentación se debe a que, como consecuencia de la actividad metabólica del microorganismo, se degradan las estructuras que confieren protección a los compuestos activos, lo que promueve una liberación controlada de los mismos y la síntesis de otros con mayor actividad. En relación con la viabilidad de los probióticos en los alimentos, esta puede verse afectada por varios factores, tales como el nivel de oxígeno en el

alimento, la permeabilidad al oxígeno del empaque, el tiempo de fermentación, la temperatura de almacenamiento, la presencia de ácidos orgánicos y otras sustancias inhibidoras que se producen durante el proceso de fermentación, etc.

En este apartado se presentan los resultados obtenidos en los estudios conducentes a la obtención de un líquido de impregnación a partir de zumo de clementina comercial con elevado contenido en compuestos antioxidantes y en unidades formadoras de colonias de *L. salivarius* CECT 4063. En forma de dos artículos científicos, se analiza el efecto que la concentración de trehalosa (0-20%, p/p) y la presión de homogeneización (0-150 MPa) ejercen sobre las propiedades antioxidantes y el crecimiento de *L. salivarius* spp. *salivarius* en zumo de clementina comercial, así como sobre la capacidad del microorganismo para inhibir al patógeno *Helicobacter pylori*, para adherirse a la mucosa intestinal y para resistir el proceso de digestión *in vitro*. También se evalúa cómo la propia fermentación con el lactobacilo afecta al contenido en compuestos con actividad antioxidante del zumo, en mayor o menor medida dependiendo de la adición o no de un 10% en peso de trehalosa al medio y/o de la homogeneización a 100 MPa del zumo fermentado y sin fermentar. Por último, se estudia la evolución de las propiedades antioxidantes y microbianas de los zumos fermentados durante su almacenamiento en refrigeración, así como su habilidad para ser incorporados en la matriz estructural de láminas de manzana (var. *Granny Smith*) mediante la técnica de impregnación a vacío. A continuación, se detallan los resultados más relevantes:

- El aumento en la concentración de trehalosa supuso un descenso notable en la actividad del agua del zumo de clementina, lo que afectó de forma negativa al crecimiento de *Lactobacillus salivarius* CECT 4063. Sin embargo, la aplicación de presiones de homogeneización inferiores a 100 MPa al zumo de clementina fermentado durante 24 h con el citado microorganismo supuso un aumento en los recuentos microbianos, lo que resultó especialmente acusado al aumentar la concentración de trehalosa presente en el medio.
- La sola adición de trehalosa al zumo de clementina disminuyó significativamente su contenido en fenoles y flavonoides totales, que aumentó tras la fermentación durante 24 h con *Lactobacillus salivarius* CECT 4063 y alcanzó valores similares a los del zumo fermentado que no incluía trehalosa en su composición. La posterior homogeneización del zumo fermentado supuso un notable descenso en el tamaño medio de las partículas presentes en el zumo y favoreció la liberación de los compuestos fenólicos de la fracción de pulpa insoluble al medio. Esto aceleró su degradación en los zumos que no incluían trehalosa en su composición, pero aumentó su biodisponibilidad en los que incluían un 10% (p/p) de trehalosa.
- La homogeneización del zumo a 100 MPa tras 24 h de fermentación resultó en un contenido en hesperidina, narirrutina y didimina significativamente más elevado, pero su efecto negativo sobre la viabilidad del probiótico hace desaconsejable su aplicación.
- Pasadas 24 h desde el inicio de la fermentación tanto los recuentos microbianos como las propiedades antioxidantes de los zumos comenzaron a disminuir, en

mayor o menor medida dependiendo de la adición de trehalosa y/o de la aplicación de altas presiones de homogeneización.

- La homogeneización a 100 MPa del zumo de clementina antes de la inoculación con *Lactobacillus salivarius* CECT 4063 mejoró notablemente el crecimiento microbiano, pero aceleró la degradación de los compuestos antioxidantes.
- La adición de un 10% (p/p) de trehalosa al zumo de clementina empleado como medio de crecimiento de *Lactobacillus salivarius* CECT 4063 aumentó notablemente su supervivencia a los jugos gástricos, pero redujo a la mitad su supervivencia a los fluidos intestinales. Esta habilidad de la trehalosa para incrementar la tolerancia del microorganismo a las condiciones ácidas del estómago se vio mermada con la aplicación de altas presiones de homogeneización al zumo fermentado que, por si sola, también resultó una técnica adecuada para mejorar la supervivencia microbiana durante la etapa gástrica y mantener invariables los recuentos durante la etapa intestinal.
- El empleo de zumo de clementina comercial como medio para el crecimiento de *Lactobacillus salivarius* CECT 4063 no mejoró significativamente su habilidad para inhibir el crecimiento del patógeno *Helicobacter pylori* pero sí su capacidad para adherirse a la mucina, el colágeno y la albúmina de suero bovino, no observándose en este caso efecto significativo alguno debido a la adición de trehalosa a la formulación del zumo y/o a la aplicación de altas presiones de homogeneización.
- Se ha observado que, debido a su menor tamaño medio de partícula, los zumos homogeneizados mostraron mayor capacidad para ser incluidos en la matriz estructural de láminas de manzana mediante la técnica de impregnación a vacío, incluso habiendo incorporado trehalosa en su composición.
- Una vez elaborado el líquido de impregnación, éste no debería almacenarse durante más de 15 días en refrigeración para mantener los recuentos por encima de 10^7 UCF/mL y conservar su condición de probiótico.

  La homogeneización a 100 MPa del zumo fermentación durante 24 h con *Lactobacillus salivarius* CECT 4063 resultó ser la mejor opción para reducir la pérdida de viabilidad del citado microorganismo tras 15 días de almacenamiento en refrigeración; sin embargo, las propiedades antioxidantes se vieron mejor preservadas al incorporar un 10% (p/p) de trehalosa al zumo de clementina previamente a su inoculación con el citado microorganismo.

En conclusión, se puede afirmar que la fermentación del zumo de clementina comercial con *Lactobacillus salivarius* CECT 4063 durante 24 h es una herramienta útil para mejorar sus propiedades antioxidantes, al mismo tiempo que le confiere un potencial efecto probiótico. En general, ni el estrés osmótico debido a la presencia de trehalosa en el medio de crecimiento ni el estrés debido al gradiente de presión que supone la aplicación de altas presiones de homogeneización mejoraron notablemente la concentración microbiana en el zumo, la adhesión del lactobacilo al epitelio intestinal o su capacidad para inhibir el crecimiento de *Helicobacter pylori*. Sin embargo, cada una de estas técnicas por separado sí logró aumentar la supervivencia de *Lactobacillus*

salivarius CECT 4063 al proceso de digestión simulada *in vitro* y al almacenamiento en condiciones de refrigeración. Del mismo modo, es de esperar que estas técnicas contribuyan a mejorar la resistencia del microorganismo durante las etapas posteriores de procesado implicadas en la obtención de un snack con potencial efecto probiótico.

ARTÍCULO 1

IMPROVING ANTIOXIDANT PROPERTIES AND PROBIOTIC EFFECT OF CLEMENTINE JUICE INOCULATED WITH *LACTOBACILLUS SALIVARIUS* SPP. *SALIVARIUS* (CECT 4063) BY TREHALOSE ADDITION AND/OR SUBLETHAL HOMOGENIZATION

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Abstract: This study evaluates the effect of trehalose addition (10 or 20%, w/w) and/or sublethal homogenization (25-150 MPa) on antioxidants content (vitamin C, total phenols, and flavonoids) and activity (measured both by ABTS-TEAC and DPPH assays), as well as on microbial counts and survival to *in vitro* digestion of clementine juice inoculated with *Lactobacillus salivarius* spp. *salivarius*. Particle size, vacuum impregnation parameters and anti-*Helicobacter pylori* effect were also measured. Incubation with the probiotic improved the antioxidant properties of the juice. Homogenization pressures below 100 MPa following incubation increased both the probiotic counts in the juice and its antioxidants bioaccessibility. Adding 10% (w/w) of trehalose to the juice was effective in preventing these bioactive compounds deterioration under adverse conditions. Once homogenized, liquids containing 10% (w/w) of trehalose became as able as those without trehalose to enter a food solid matrix. Inhibition of *Helicobacter pylori* growth was evident in all probiotic beverages.

Keywords: trehalose, homogenization, probiotic, *in vitro* digestion, antioxidants, anti-*Helicobacter pylori*

1. Introduction

Research carried out in recent years demonstrates the close relationship existing between gut microbiota and the incidence of certain diseases, whether infectious, degenerative, metabolic, or psychological (Pirbaglou et al., 2016; Rouxinol-Dias et al., 2016; Siong et al., 2015). There is also evidence of the beneficial effect that food matrices exert on the growth and survival of certain microorganisms during gastric transit, thus conditioning the composition of human microbiota and making probiotics consumption more recommendable as part of a food than in the form of supplements (Ranadheera et al., 2010).

Fermented dairy foods are commonly used as probiotic carriers since they are rich in proteins and lipids that protect them against the adverse conditions of the digestive

system (Khan, 2014). However, the consumption of such products is restricted in individuals with lactose intolerance and/or with high cholesterol levels, as well as in the population following vegetarian or vegan diets. This has encouraged the recent use of alternative food matrices for the delivery of probiotics (Anekella & Orsat 2013; Chen & Mustapha, 2012; Furtado-Martins et al., 2013; Rivera-Espinoza & Gallardo-Navarro, 2010). In particular, fruit and vegetable juices have been suggested as an ideal medium for cultivating probiotic microorganisms since they inherently contain beneficial nutrients such as minerals, vitamins, dietary fibber, antioxidants, and they have taste profiles that are pleasing to all the age groups (Rivera-Espinoza & Gallardo-Navarro, 2010). However, these food matrices do not always fulfil the pH, or the essential amino acids and vitamins required for the optimum growth of most LAB with proven probiotic effect. It is therefore necessary, apart from selecting the appropriate strain for each substrate, adding certain supports (prebiotics, cryoprotectants, soy germ powder, yeast extract, etc.) and/or applying some processing technologies (microencapsulation, vacuum impregnation, sublethal homogenization, etc.) in order to meet the minimum concentration of 10^6-10^7 viable probiotic cells per millilitre or gram of product at the expiration date, which is required to make an EU-based health claim (Rad et al., 2013).

In an attempt to develop a probiotic apple snack with effect against the infection caused by *Helicobacter pylori*, Betoret et al. (2012b) analysed the growth of two different strains with proven anti-*Helicobacter* activity (*Lactobacillus salivarius* spp. *salivarius* CECT 4063 and *Lactobacillus acidophilus* CECT 903) in two fruit juices (mandarin juice and pineapple and grape juice) with organoleptic properties similar to those of fresh apple. This study concluded that the highest counts (8.44 log CFU/mL) were obtained after mandarin juice fermentation at pH ≈ 6 with *Lactobacillus salivarius* spp. *salivarius* for 24 h. The further vacuum impregnation of apple slices with this liquid and their subsequent drying with air at 30 °C resulted in a probiotic snack (7.98 log CFU/g) potentially effective against *Helicobacter pylori* infection, but that need to be improved in order to please the final consumer and to increase its shelf life. To achieve these effects without negatively affecting the probiotic content, adding trehalose to clementine juice before fermentation and/or homogenizing the fermented juice could be good options.

Trehalose is a very important natural metabolite and a prospective food additive. This non-reducing sugar is known to protect cell membranes and proteins against abiotic stress caused by heat, exposure to ethanol or high concentration of solutes in dehydration processes (Mansure et al., 1994; Nery et al., 2008; Ohtake & Wang, 2011; Wolkers et al., 2001). Therefore, trehalose has broad biotechnological applications, especially as a preservative of food and biological products, but also as a sucrose substitute in bakery and pastry products with an almost flat response on blood glucose levels (Ohtake & Wang 2011). When added to mandarin juice before its inoculation with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 in a 100 g/kg ratio, trehalose hardly affected the microbial growth and significantly improved the probiotic survival in the juice after 10 days of refrigerated storage (Betoret et al., 2017).

High pressure homogenization (HPH) is a unit operation traditionally employed in juice manufacturing as an alternative to thermal treatment for microbial inactivation. According to recent studies, this new technology has been proven to enhance the survival and the overall functionality of certain probiotic strains when applied at moderate levels (Tabanelli et al., 2012). In particular, sublethal HPH has been reported to enhance organoleptic and functional properties of probiotic fermented milks and cheeses by improving strain viability over refrigerated storage and accelerating fermentation kinetics (Burns et al., 2015; Patrigniani et al., 2009); Lanciotti et al. (2007) also showed that HPH not exceeding 150 MPa was able to modify both the fermentation kinetics and the enzymatic activities of starter and non-starter lactic acid bacteria without detrimental effects on cell viability. When applied to low pulp mandarin juice, the homogenization in the range between 5 and 30 MPa significantly reduced the average size of suspended solids without negatively affecting the antiradical activity and the content of bioactive compounds (Betoret et al., 2012a). Therefore, when using the homogenized juice as impregnating solution, more liquid and more functional compounds are expected to be introduced into the structural matrix of the impregnated sample. Beyond these effects of mainly technological interest, non-lethal HPH was observed to increase the hydrophobicity in trials with different probiotic strains, which is directly related to their capacity of adhesion to the intestinal cells and their resistance to the digestion process (Basson et al., 2007; Betoret et al., 2017; Tabanelli et al., 2012). Although there are some previous studies about the effect of high-pressure processing and trehalose addition on functional properties of citrus juices enriched with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 (Betoret et al. 2012b & 2017), none of them delves into the effect of fermentation with the probiotic on the antioxidant properties of the juice. Moreover, since benefits attributed to probiotics do not only depend on the amount ingested, it is also important to evaluate how microbial properties such as the tolerance to acids and bile exposure or the antimicrobial activity are affected under such processing conditions.

According to everything commented above, the present study aims to evaluate the effect of trehalose addition (10 or 20% by weight) and/or sublethal homogenization (25, 50, 100 or 150 MPa) on some physicochemical (water activity, ºBrix, pH, particle size, vacuum impregnation parameters, main antioxidants content) and microbial properties (microbial counts, survival to *in vitro* digestion and anti-*Helicobacter pylori* effect) of clementine juice inoculated with *Lactobacillus salivarius* spp. *salivarius* CECT 4063.

2. Materials and methods

2.1. Raw materials and bacterial cultures

To carry out this study, *Granny Smith* variety apples, commercial clementine juice (Hacendado trademark) and food-grade trehalose obtained from tapioca starch (TREHATM, Cargill Ibérica, Barcelona, Spain) were employed. *Lactobacillus salivarius* spp. *salivarius* strain CECT 4063 and *Helicobacter pylori* strain NCTC 11637 also used in this research were supplied by the Spanish Culture Type Collection (Paterna, España) and the National Collection of Type Cultures (Public Health England, UK), respectively.

Stock cultures were prepared by adding sterile glycerol (20% v/v) to the activated culture and freezing at -20 °C in sterile screwcap cryovials. When necessary, glycerol was removed from the thawed stock culture by centrifugation at 3000 rpm and 4 °C for 1 min. Then, probiotic stock cultures were subcultured onto Man Rogosa and Sharp (MRS) broth (Scharlab, S.L., Barcelona, Spain), while pathogenic stock cultures were plated on Columbia Agar Base supplemented with 10% (v/v) of defibrinated horse blood (Scharlab, S.L., Barcelona, Spain). After 24 h of incubation at 37 °C under anaerobic conditions using AnaeroGen sachets (Oxoid Ltd., Basingstoke, UK), a probiotic cell density of (2.8 ± 0.8) x 10^9 CFU/mL was reached. In the case of *Helicobacter pylori*, total biomass obtained after 48 h of incubation at 37 °C under anaerobic conditions using AnaeroGen sachets (Oxoid Ltd., Basingstoke, UK) was resuspended in Brucella broth (Scharlab, S.L., Barcelona, Spain) until reaching a concentration around 10^9 CFU/mL, corresponding to a value of 6 on the McFarland optical density scale.

2.2. Probiotic juices preparation and homogenization procedure

According to the procedure described by Betoret et al. (2012b), commercial clementine juice pH was increased to 6.5 by adding around 9.8 g/L of sodium bicarbonate. This was intended to bring the pH of the juice close to the optimum for the lactic-acid bacteria growth. Also, with the purpose of reaching the highest microbial content after incubation, 5 g of freeze-dried yeast extract were incorporated per litter of juice. When necessary, trehalose was added at a rate of 10 or 20 grams per 100 grams of juice prior to its inoculation with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 to get an initial concentration of 7.03 ± 0.13 log CFU/mL. After incubation for 24 h at 37 °C to achieve the stationary phase, part of the juice-based probiotic liquids were submitted to homogenization at 25, 50, 100 or 150 MPa in a laboratory scale high pressure homogenizer (Panda Plus 2000, GEA-Niro Soavi, Parma, Italy). Homogenized and non-homogenized (0 MPa) juices were then kept refrigerated at 4 °C until analysis.

2.3. Analytical determinations

All the analytical determinations described in this section were performed at least in triplicate and, depending on the variable being analysed, within 24 h after the incubation and/or the homogenization step.

2.3.1. a_w, ºBrix, pH, particle size and vacuum impregnation parameters.

Water activity (a_w) was measured with an accuracy of ± 0.003 in a dew point hygrometer (Decagon Agualab, model CX-2), previously calibrated with reference saturated salts. Total soluble solids content expressed in ºBrix was measured in a table refractometer (Abbe Atago, model Nar-T3) at the constant temperature of 20 °C. The pH was measured in a potentiometer provided with a temperature self-calibrating system (Mettler Toledo, model S20 SevenEasyTM), previously calibrated with buffer solutions at pH 4 and 7. The size of the particles was analysed in a Malvern Mastersizer analyser (Malvern Instruments Ltd, model 2000) in a measuring range between 0.02 and 1000 microns. Values employed for the refractive cloud and the dispersed phase indices were 1.73 and 1.33 respectively, while that set for the absorption of the cloud particles index was 0.1

(Corredig et al., 2001). Apart from the corresponding distribution curves of the particle size, also the mean diameter over volume (D[4,3]) and the particle diameter at 90% in the cumulative distribution (d_{90}) were obtained for each sample. Finally, the ability of the different juices to be employed as impregnating solutions in a vacuum impregnation process was analysed. For this purpose, vacuum impregnation trials were conducted in a pilot plant equipment specially designed for taking the weight measures necessary for the characteristic impregnation parameters calculation (Salvatori et al., 1998): the volume fraction of the solid matrix that was filled with the impregnating solution after the vacuum stage (X_1, in m^3 solution/m^3 fresh sample) and that after the atmospheric stage (X, in m^3 solution/m^3 fresh sample); the relative volumetric deformation undergone by the solid matrix after the vacuum step (γ_1, dimensionless) and that after the atmospheric step (γ, dimensionless); and the effective porosity (ε, dimensionless). *Granny Smith* apple rings (65 mm outer diameter, 20 mm inner diameter and 5 mm thick) were employed as solid matrix. The working conditions were set at 50 mbar for 10 min and atmospheric pressure for 10 min more, as in Betoret et al. (2012b).

2.3.2. Antioxidant properties

Vitamin C content was determined by potentiometric titration with a 0.005 M chloramine-T solution and a platinum electrode (Metrohm 800 dosino Ti Application note nº T30). Sample preparation was performed by mixing 15 mL of juice with 50 mL of distilled water, 2 mL of sulfuric acid 2 M and 10 mg of potassium iodide. A 350-ppm ascorbic solution was used for standardizing the titrant solution. Results were expressed in mg of ascorbic acid per mL (mg AA/mL).

Total phenols content was determined by the Folin-Ciocalteu method (Singleton & Rossi, 1965), which is based on measuring at 760 nm the intensity of the blue colour that appears by reaction at basic pH between the Folin-Ciocalteu reagent (mixture of phosphor wolfram and phosphor molybdic acids) and those phenols present in the sample. For carrying out measurements, 125 μL of sample diluted in bidistilled water at a ratio 1:5 (v/v) were mixed in a spectrophotometer cuvette with 125 μL of the Folin-Ciocalteu reagent and 500 μL of bidistilled water. After 6 min in darkness, 1.25 mL of a 7.5% (w/v) sodium carbonate solution and 1 mL of bidistilled water were added. Absorbance was measured after 90 min of reaction at 760 nm in a Thermo Scientific Helios Zeta UV/Vis spectrophotometer. A white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. Results obtained were compared with a gallic acid standard and expressed in mg of gallic acid equivalents per mL of sample (mg GAE/mL).

Total flavonoids content was determined by the colorimetric method proposed by Luximon-Ramma et al. (2005). In this case, 1.5 mL of sample diluted in bidistilled water at a ratio 1:5 (v/v) were mixed in a spectrophotometer cuvette with 1.5 mL of a 2% (w/v) in methanol aluminium chloride solution. Absorbance was measured after 10 min of reaction in darkness at 368 nm in a Thermo Scientific Helios Zeta UV/Vis spectrophotometer. A white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. Results obtained were compared with

a quercetin standard and expressed in mg of quercetin equivalents per mL of sample (mg QE/mL).

Antioxidant capacity of juice samples was analysed both by the ABTS-TEAC and the DPPH assays. ABTS-TEAC assay is based on measuring ABTS- cation discoloration from green-blue to colourless when reduced by the antioxidant compounds of the sample. In order to produce the ABTS- cation, a solution containing 7 mM of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and 2.45 mM of potassium persulfate in bidistilled water was incubated in darkness for 16 h. Once the radical was released, it was diluted in phosphate buffer until an absorbance of 0.7 ± 0.01 at 734 nm was reached (Re et al., 1999). Then, 2910 μL of this solution was mixed with 90 μL of diluted sample (1 mL of juice in 9 mL of bidistilled water). Absorbance was in this case measured after 6 min of reaction in darkness at 734 nm in a Thermo Scientific Helios Zeta UV/Vis spectrophotometer. A white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. Results obtained were compared with a Trolox standard and expressed in mg of Trolox equivalents per mL of sample (mg TE/mL).

DPPH assay measures the colour change from purple to yellow that takes place when a solution containing radical DPPH (2,2-diphenyl-1-picrylhydrazyl) is reduced by the antioxidant compounds of the sample (Brand-Williams et al.,1995; Molyneux, 2004). To achieve this, 100 μL of diluted sample (1 mL of juice in 4 mL of distilled water), 900 □L of spectrophotometric grade methanol (purity ≥ 99%) and 2000 μL of a 100 mM solution of DPPH in methanol were mixed in a spectrophotometer cuvette. Absorbance was measured after 30 min of reaction in darkness at 517 nm in a Thermo Scientific Helios Zeta UV/Vis spectrophotometer. As previously mentioned, a white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. Results obtained were compared with a DPPH standard and, according to the initial amount of DPPH poured into the cuvette, expressed in mg of reduced DPPH per mL of sample (mg $DPPH_{red}$/mL).

2.3.3. Microbial counts

Lactobacillus salivarius spp. *salivarius* content in inoculated juices was estimated by the serial dilution in sterile peptone water and plating procedure. As reported in Collado & Hernández (2007), Lactobacilli MRS Agar plates were incubated at 37 °C for 2 days under anaerobic conditions using AnaeroGen sachets (Oxoid Ltd., Basingstoke, UK).

2.3.4. *In vitro* gastrointestinal simulation

In order to evaluate the potential probiotic resistance to the intestinal and gastric juices, a modification of the protocol proposed by García-Hernández et al. (2018) for *in vitro* simulation of the gastrointestinal process was employed. To simulate gastric digestion stage, a sterile saline solution (0.5%, w/v) containing 3 g of pepsin isolated from porcine gastric mucosa (Sigma Life Science) and adjusted to pH 2 with 0.5 N hydrochloric acid was employed. To simulate intestinal digestion stage, a sterile saline solution (0.5%, w/v) containing 1 g of pancreatin isolated from porcine pancreas (Sigma Life Science)

adjusted to pH 8 with 0.1 N NaOH solution was used. Since the analysed samples had no fat in its composition, no bile salts were included in the intestinal simulation stage. Throughout all the process, samples were stirred at 100 rpm in an orbital incubator (Ivymen System) placed inside an incubation oven at 37 °C. In the first place, 20 mL of juice were mixed with 70 mL of pepsin solution in an Erlenmeyer flask. Microbial counts were performed at different times throughout the process (0, 10, 30, 60 y 120 min) by the dilution and plating procedure described above. Then, 56 mL of the residual liquid obtained after the gastric stage were mixed with 30 mL of pancreatin solution. In this case, microbial counts were performed at the beginning and at 30, 60, 120, 240 y 360 min from the start of the stage by the dilution and plating procedure describe above. The viabilities of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 after both the gastric step (%$VIAB_{ST1}$) and the intestinal step (%$VIAB_{ST2}$) were calculated as the ratio in percentage between the number of living cells leaving and entering each step. In accordance with this, the probiotic resistance to the entire simulated gastrointestinal digestion (%$VIAB_{TOTAL}$) was calculated from the product of probiotic viabilities obtained for each of the two steps making up the process (García-Hernández et al., 2012).

2.3.5. Anti-*Helicobacter pylori* effect

The inhibitory effect of *Lactobacillus salivarius* spp. salivarius strain CECT 4063 on the growth of *Helicobacter pylori* strain NCTC 11637 was tested by an agar diffusion method, similar to that described by Lin et al. (2011). To this end, aliquots of 100 μL of the pathogen dilution corresponding to a value of 6 on the McFarland optical density scale were spread on Columbia Agar Base plates supplemented with 10% (v/v) of defibrinated horse blood (Scharlab, S.L., Barcelona, Spain). Wells (8 mm in diameter) were punched in each agar plate using a sterile stainless-steel borer. Discs of the same size obtained from MRS agar plates seeded with 1 mL of a particular probiotic juice and incubated at 37 °C for 24 h under anaerobic conditions were place into the wells. Discs obtained by the same procedure from MRS agar plates seeded with 1 mL of the MRS broth with a probiotic cell density of (2.8 ± 0.8) x 10^9 CFU/mL were used as control. The plates were then incubated at 37 °C for 48 h under microaerophilic conditions. After that, the diameters of the inhibition zones around the wells were measured. Results were expressed as the mean diameter of triplicate independent experiments for each sample.

2.3.6. Statistical analysis

The statistical significance degree of the different variables considered on the results obtained was evaluated with the Statgraphics Centurion XVI program by means of simple and multivariate analysis of variance with a 95% of confidence level.

3. Results and discussion

3.1. Effect of processing variables on a_w, pH, °Brix and particle size of clementine juice

Main physicochemical properties of the different liquids prepared from commercial clementine juice are shown in Table 1.

Table 1. Physicochemical properties of clementine juice at pH 6.5 non-inoculated (NI) and inoculated and incubated for 24 h at 37 °C with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 as a function of trehalose concentration and/or the homogenization pressure.

%TREH	P (MPa)	a_w	pH	Brix	D[4,3]*	d_{90}**
0	NI	0.9856 (0.0009)^{fg}	6.567 (0.012)^{i}	12.07 (0.06)^{a}	227 (24)^{de}	572 (72)^{d}
	0	0.9863 (0.0003)^{g}	4.93 (0.06)^{a}	12.0 (0.2)^{a}	232 (8)^{de}	577 (6)^{d}
	25	0.9866 (0.0011)^{g}	4.94 (0.06)^{ab}	12.03 (0.10)^{a}	41 (4)^{a}	100 (6)^{a}
	50	0.9862 (0.0012)^{g}	4.94 (0.10)^{ab}	12.0 (0.2)^{a}	38 (5)^{a}	93 (8)^{a}
	100	0.9865 (0.0016)^{g}	4.97 (0.12)^{b}	11.97 (0.14)^{a}	28.4 (0.8)^{a}	79 (2)^{a}
	150	0.9858 (0.0005)^{fg}	4.96 (0.10)^{ab}	11.77 (0.10)^{a}	43 (3)^{a}	101 (4)^{a}
10	NI	0.9803 (0.0008)^{c}	6.770 (0.010)^{j}	20.2 (0.4)^{c}	238 (20)^{e}	597 (54)^{d}
	0	0.9818 (0.0010)^{cd}	5.45 (0.03)^{c}	19.27 (0.15)^{c}	211 (21)^{bc}	530 (63)^{bc}
	25	0.984 (0.002)^{ef}	5.51 (0.06)^{def}	18.60 (0.10)^{b}	38.3 (1.1)^{a}	107 (3)^{a}
	50	0.9828 (0.0012)^{de}	5.49 (0.03)^{d}	18.60 (0.10)^{b}	50 (3)^{a}	90 (3)^{a}
	100	0.9836 (0.0008)^{e}	5.49 (0.17)^{d}	18.87 (0.15)^{b}	29 (3)^{a}	75 (5)^{a}
	150	0.9834 (0.0006)^{de}	5.50 (0.02)^{de}	18.90 (0.10)^{b}	27.8 (0.8)^{a}	69.2 (1.1)^{a}
20	NI	0.9726 (0.0010)^{a}	6.803 (0.006)^{k}	28.13 (0.06)^{g}	206 (13)^{b}	522 (31)^{b}
	0	0.9767 (0.0004)^{b}	5.55 (0.04)^{gh}	21.90 (0.10)^{d}	221 (11)^{cd}	563 (29)^{cd}
	25	0.9782 (0.0003)^{b}	5.53 (0.03)^{fgh}	21.14 (0.10)^{d}	34.3 (0.9)^{a}	99 (2)^{a}
	50	0.9777 (0.0003)^{b}	5.54 (0.02)^{ghi}	25.2 (1.1)^{f}	31 (2)^{a}	86 (3)^{a}
	100	0.9773 (0.0010)^{b}	5.55 (0.03)^{gh}	24.70 (0.10)^{e}	28 (3)^{a}	76 (4)^{a}
	150	0.9779 (0.0002)^{b}	5.57 (0.02)^{h}	24.93 (0.15)^{ef}	27 (4)^{a}	71 (8)^{a}

Mean values and standard deviation in brackets.

* mean diameter over volume in microns.

** particle diameter at 90% in the cumulative distribution in microns.

$^{abc...}$ different superscripts in the same column indicate statistically significant differences ($p < 0.05$)

Comparing data obtained for those juices only formulated with sodium bicarbonate and trehalose before their inoculation with the probiotic microorganism (NI samples), the effect of such disaccharide concentration have been evidenced. As expected, increasing the trehalose content from 0 to 20% (w/w) resulted in significant changes in Brix, pH and water activity values but hardly affected the size of the particles present in the juice. In particular, ºBrix values of non-inoculated liquids significantly increased as it did the amount of trehalose present in the juice, thus reducing the water activity values and promoting osmotic stress with negative effect on microbial growth. As regards the pH, it significantly increased with the amount of trehalose added to the juice formulation. Since trehalose is known to stabilize biological systems submitted to adverse conditions by hydrogen bonding to proteins, thus raising and modulating cellular pH levels towards a lower acid level and a lower cellular stress (Kaushik† & Bhat, 2003), no wonder its ability to also increase the pH of clementine juice. Finally, it should be mentioned that, since the disaccharide concentration in the juice was considerably below its maximum solubility in water at 20 °C (reported in 2009 by Jain & Roy to be 68,9 g/100 g), neither the mean diameter over volume (D[4,3]) nor the particle diameter at 90% in the cumulative distribution (d_{90}) were notably affected by the addition of trehalose (Fig. 1a). The mean diameter over volume was around 223 ± 13 μm and the size of the 90% of the particles present in the juice was of the order of 560 ± 29 μm.

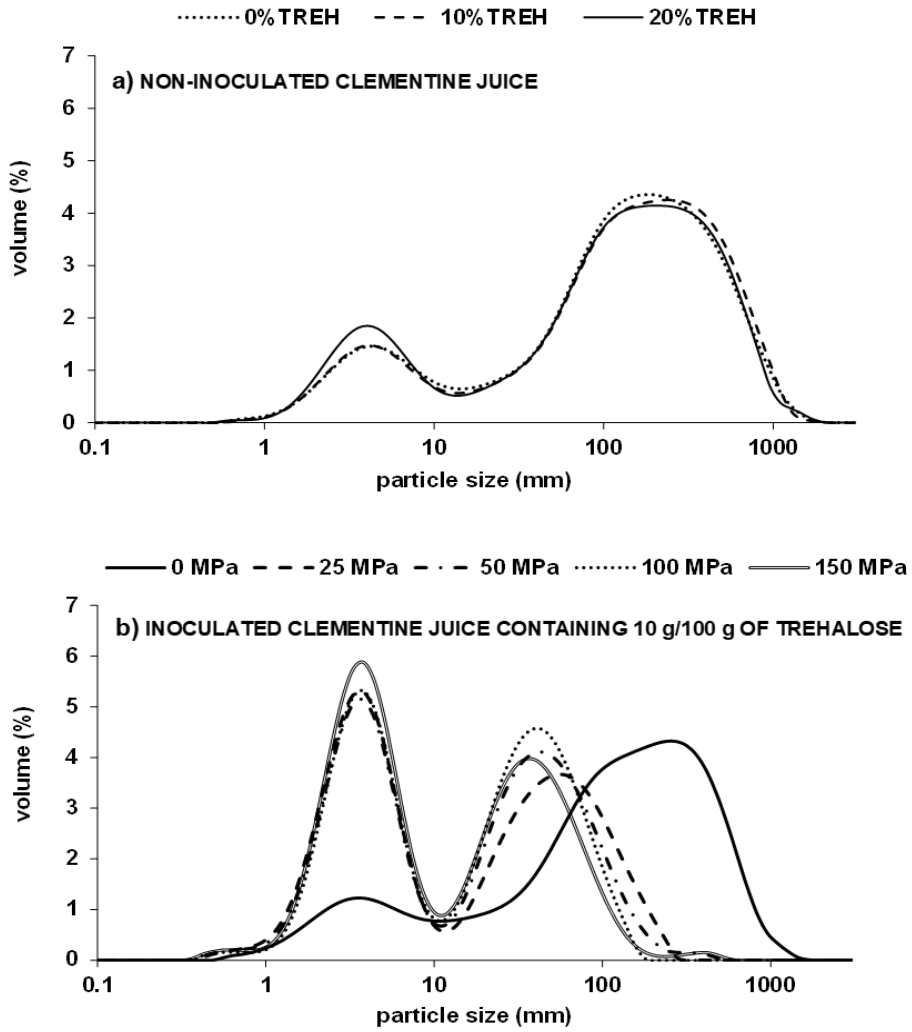


Figure 1. Clementine juice distribution curves for particle size as affected by trehalose concentration (a) and the homogenization pressure (b).

Also, from values reported in Table 1 it is possible to realize the effect of the juice incubation with the probiotic microorganism on its main physicochemical properties. What is more outstanding is the sharp decline in pH values, and to a lesser extent in the soluble solids content, as a result of lactic acid production by *Lactobacillus salivarius* spp. *salivarius* CECT 4063 from the available nutrients. Because, as it will be discussed below, the growth of the selected microorganism is adversely affected by increasing the osmolality of the growing media, the pH drop linked to 24 h incubation was less marked as it increased from 0 to 20% by weight the concentration of trehalose in the juice. None of the parameters related to the particles size were noticeably modified by the probiotic growth in the juice, but yes as a result of the application of sub lethal homogenization pressures. According to data shown in Table 1, the homogenization in the range of pressures between 25 and 150 MPa caused, regardless of the pressure applied, a significant decrease in the size of the particles present in the juice. This reduction in both the mean diameter over volume (D[4,3]) and the particle diameter at 90% in the

cumulative distribution (d_{90}) is confirmed in the corresponding distribution curves for particle size (Fig. 1b). As it can be observed, all the analysed liquids showed a bimodal distribution between 1 and 1000 μm before homogenization (irrespective of having been previously inoculated or not) and between 1 and 200 μm after homogenization. This considerable decrease in the size of the suspended particles resulting from the homogenization step would imply, as reported by Betoret et al. (2012a), an increase in the cloud stability and in the juice incorporation into a food matrix by means of vacuum impregnation without negatively affecting its antiradical activity. However, the homogenization in the range of pressures between 25 and 150 MPa had not significantly effect on a_w, pH and Brix values of clementine juice containing the probiotic microorganism.

3.2. Effect of processing variables on *Lactobacillus salivarius* spp. *salivarius* CECT 4063 growing in clementine juice

Figure 2 shows the effect that the trehalose concentration (from 0 to 20 g/100 g) and/or the homogenization pressure (from 0 to 150 MPa) had on the microbial growth of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 in clementine juice at pH 6.5. It should first be noted that incubation resulted in a general rise in the microbial population from $(1.1 \pm 0.3) \times 10^7$ CFU/mL to at least $(1.8 \pm 0.5) \times 10^8$ CFU/mL, which increased even further after homogenization. Therefore, all liquids showed a microbial content over 10^7 CFU/mL, which is the limit established at the time of consumption by the International Dairy Federation to state that a food has probiotic properties (Manojlović et al., 2010).

Regarding the effect of the above-mentioned variables, multifactor analysis of variance showed, at the 95% confidence level, that both the homogenization pressure and the trehalose concentration, as well as the interaction between them, significantly affected the probiotic viability (Fig. 2b). For not homogenized juices (0 MPa), the increase in the trehalose concentration from 0 to 20% (w/w) significantly diminished the number of viable cells. Certainly, as evidenced in Table 1, the lower water activity achieved by the juice as the trehalose concentration increased might induce osmotic stress situations that restricted the growth of the probiotic microorganism. However, differences in the probiotic content due to the amount of disaccharide added to the juice were mildly attenuated by the application of a homogenization step. As evidenced in Fig. 2, microbial counts for a given trehalose content increased with the homogenization pressure up to a maximum around 50 MPa, when the counts started to decline. This result is comparable to that obtained by Tabanelli et al. (2013) on different lactic acid bacteria strains commonly used in commercial dairy products when submitted to sublethal high pressure homogenization treatments. In his study, Tabanelli et al. (2013) verified that the viability of *L. paracasei* A13, *L. acidophilus* 08 Dru+ and *L. delbrueckii* subsp. *lactis* 200 and their bile-resistant derivatives *L. acidophilus* Dru+ and *L. delbrueckii* subsp. *lactis* 200+ suspended in PBS was reduced in less than 0.2 Log CFU/mL by the homogenization at 50 MPa. The increase in the number of living cells observed in the present study when homogenizing in the range 0-50 MPa would highlight the relevance of the matrix effect, so that the viability of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 was enhanced

in the presence of clementine juice. This finding was previously supported by other authors (Chaikham, 2015; Sagdic et al., 2012; Shah et al., 2010), who attributed to the high content in antioxidant compounds of certain products, as it is the case of clementine juice, the ability to create a favourable anaerobic environment for probiotic survival.

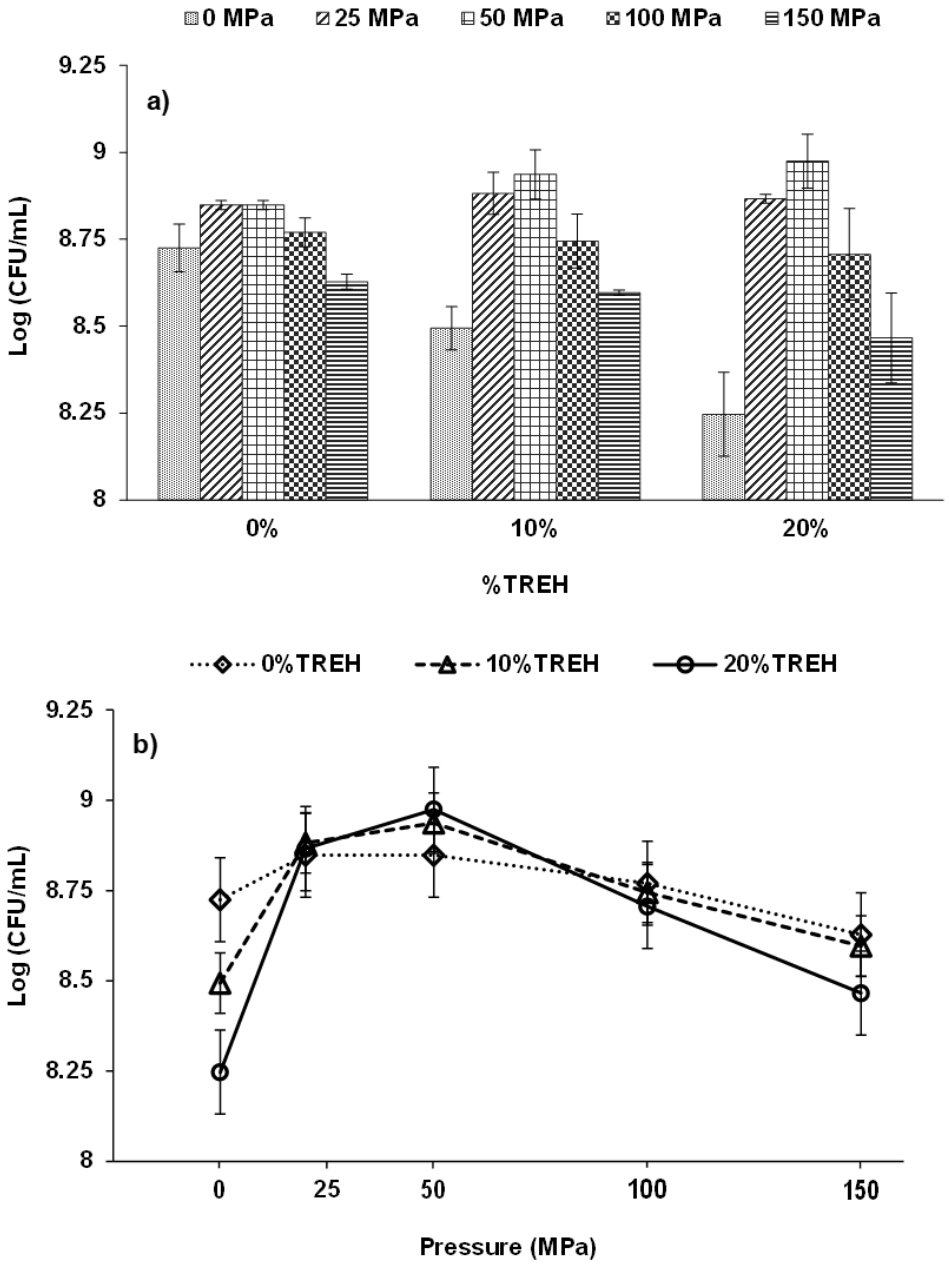


Figure 2. *Lactobacillus salivarius* spp. *salivarius* CECT 4063 growth as affected by trehalose concentration and/or the homogenisation pressure: mean values and standard deviation as error bars (a) and graph of interactions and LSD intervals with a 95% confidence level(b).

Also, adding trehalose to the juice involved slight increase in the viable counts after homogenization at 50 MPa, thus evidencing its role in preserving proteins and biological membranes under moderate stressing conditions (Atarés et al., 2009; Betoret et al., 2014; Crowe et al., 1984; Lins et al., 2004). However, the increase in the trehalose

concentration from 0 to 20 g/100 g had just the opposite effect when homogenization pressures above 50 MPa were applied. In other words, the beneficial effect that trehalose had on the probiotic viability was blinded by the adverse effect of the osmotic stress when coupled with high pressure gradients. Thus, in order to achieve the greatest number of living cells in clementine juice it would be advisable to apply homogenization pressures and trehalose concentrations not exceeding 100 MPa and 10% by weight, respectively.

3.3. Effect of processing variables on antioxidant properties of clementine juice

The effect of the formulation with 10% of trehalose by weight, the inoculation and incubation with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 and the homogenization at 50 or 100 MPa on main antioxidant properties analysed in the different liquids obtained from commercial clementine juice is shown in Table 2.

Starting with the ascorbic acid or the vitamin C, none of the variables considered caused significant changes in its content (p-value < 0.05). Despite being a commercial juice that may have been submitted to intense thermal processing, vitamin C content was similar to that reported in previous studies for freshly squeezed clementine juice (Bermejo & Cano, 2012; Fabroni et al., 2016). This result suggests that the juice was probably enriched with this vitamin. In any case, both natural and/or added ascorbic acid remained unchanged against the slight temperature increase (observed to be between 17 and 21 °C per 100 MPa) caused by forcing the liquid to pass through a very tight hole.

Table 2. Antioxidant properties of clementine juice at pH 6.5 non-inoculated (NI) and inoculated and incubated for 24 h at 37 ºC with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 as a function of trehalose concentration and/or the homogenization pressure.

%TREH	P (MPa)	VITAMIN C (mg AA/mL)	PHENOLS (mg GAE/mL)	FLAVONOIDS (mg QE/mL)	ABTS-TEAC (mg TE/mL)	DPPH (mg $DPPH_{red}$/mL)
0	**NI**	0.35 (0.05)^{a}	0.72 (0.03)^{b}	0.466 (0.014)^{b}	0.77 (0.07)^{a}	1.6 (0.6)^{bc}
	0	0.35 (0.02)^{a}	0.78 (0.07)^{cd}	1.01 (0.07)^{g}	1.14 (0.08)^{d}	1.52 (0.02)^{ab}
	50	0.33 (0.07)^{a}	0.754 (0.007)^{bc}	0.99 (0.03)^{fg}	0.98 (0.14)^{c}	1.5 (0.3)^{ab}
	100	0.35 (0.05)^{a}	0.724 (0.007)^{b}	0.954 (0.014)^{de}	0.95 (0.12)^{bc}	1.4 (0.3)^{ab}
10	**NI**	0.355 (0.005)^{a}	0.64 (0.05)^{a}	0.37 (0.08)^{a}	0.83 (0.12)^{ab}	1.87 (0.09)^{d}
	0	0.352 (0.008)^{a}	0.79 (0.08)^{cd}	0.904 (0.004)^{c}	1.00 (0.12)^{c}	1.7 (0.2)^{cd}
	50	0.34 (0.02)^{a}	0.85 (0.08)^{e}	0.97 (0.06)^{ef}	1.03 (0.06)^{cd}	1.39 (0.04)^{a}
	100	0.35 (0.02)^{a}	0.82 (0.08)^{de}	0.93 (0.03)^{cd}	0.8 (0.3)^{a}	1.39 (0.07)^{a}

Mean values and standard deviation in brackets.
$^{abc...}$ different superscripts in the same column indicate statistically significant differences (p < 0.05)

Regarding the total phenols and flavonoids content (Table 2), they behaved in a similar way against variations in the processing conditions, which is logical given that flavonoids are a particular type of phenols. In the case of non-inoculated juices, the addition of 10 g of trehalose per 100 g of juice resulted in a significant decrease in both total phenols and flavonoids content. This effect was previously observed by Oku et al. (2005) for unsaturated fatty acids and explained in terms of trehalose ability to prevent oxidation by joining double bounds of the acyl chains, which are also part of the chemical structure

of the most abundant flavonoids (hesperidin, narirutin and didymin) in clementine juice (Betoret et al., 2009). Therefore, the decrease in the antioxidant compounds of phenolic type content reported after the addition of trehalose to the juice composition could be due to the fact that they were more protected against the oxidation induced for their quantification.

Regarding the growth of the probiotic bacteria in the juice, it significantly increased both total phenols but specifically flavonoids content. Similar results were observed in previous studies over different food matrices: whole apple juice fermented with *Lactobacillus acidophilus* (Ankolekar et al., 2012), barley and oat flour fermented with different food-graded lactic acid bacteria (Hole et al., 2012), soybean fermented with *Bacillus pumilus* (Cho et al., 2011) and *Bacillus subtilis* (Chung et al., 2011), cowpea flour fermented with *Lactobacillus plantarum* (Dueñas et al., 2005) and papaya juice fermented with either *Lactobacillus acidophilus* or *Lactobacillus plantarum* (Ronghao et al., 2018). Apparently, the pH lowering caused by the growth of these microorganisms might activate cellulolytic, ligninolytic and pectinolytic enzymes, thus facilitating the release of phenols from their complexed form in dietary fiber into freely soluble and much more available forms (Huynh et al., 2014).

In relation to the subsequent homogenization of the incubated juice, its effect on phenols and flavonoids total content was slightly different, depending on the presence or absence of trehalose in the juice. Therefore, when 10% of trehalose by weight was added to the juice the homogenization caused, regardless of the pressure applied, a slight but significant increase in both total phenols and flavonoids content. Since phenolic compounds are known to be bounded to the juice pulp fraction, when its average size was reduced by the application of a homogenization step, total phenols contained therein would be expected to release to increase their bioavailability. However, when no trehalose was added to the juices, such phenolics release seemed to favour their faster degradation.

Finally, antioxidant activity response to the processing variables considered was different depending on the analytical method employed (Table 2). As reported by some authors (Stratil et al., 2007), the DPPH assay could be less selective than the ABTS one since the DPPH radical is only able to react with the most reactive phenols and not with less reactive and more stable ones. What is observed in the present study is that the antioxidant activity measured by the ABTS-TEAC assay was mainly affected by the microbial growing, whereas that measured by the DPPH assay was more affected by the addition of trehalose or the application of a homogenization step. Common for measurements obtained by the two different assays was the more or less evident decline in the antioxidant activity values after the homogenization at 50 or 100 MPa, especially in inoculated juices containing 10% of trehalose by weight.

3.4. Effect of processing variables on impregnating properties of clementine juice

The values of the main parameters obtained after the vacuum impregnation experiments carried out at pilot scale with 5 mm thick apple rings and the different

liquids obtained from commercial clementine juice are shown in Table 3. It is worth noting that the average volumetric impregnation parameter value (X = 0.2 ± 0.04 m^3/m^3) was similar to that obtained in previous studies using sucrose isotonic solutions (Fito et al., 2001) and apple juice or whole milk inoculated with *L. casei* spp. *rhamnosus* (Betoret et al., 2003) as impregnating liquids.

Table 3. Impregnation parameters of inoculated clementine juice as a function of trehalose concentration and/or the homogenization pressure.

%TREH	P (MPa)	γ_1	γ	X_1	X	ε_e
0	0	0.091 (0.003)^{c}	0.012 (0.007)^{b}	0.150 (0.010)^{d}	0.19 (0,02)^{ab}	0.20 (0.02)^{ab}
	50	0.03 (0.02)^{b}	0.006 (0.013)^{b}	0.052 (0.010)^{b}	0.23 (0,02)^{b}	0.24 (0.02)^{abc}
	100	-0.02 (0.02)^{a}	0.004 (0.007)^{b}	0.021 (0.010)^{a}	0.241 (0,002)^{b}	0.27 (0.04)^{bc}
10	0	0.10 (0.03)^{c}	-0.015 (0.02)^{ab}	0.090 (0.010)^{c}	0.16 (0.03)^{a}	0.18 (0.05)^{a}
	50	0.060 (0.010)^{b}	-0.06 (0.02)^{ab}	0.15 (0.02)^{d}	0.26 (0.02)^{b}	0.28 (0.05)^{c}
	100	0.049 (0.017)^{b}	-0.09 (0.04)^{a}	0.17 (0.02)^{d}	0.22 (0.07)^{ab}	0.29 (0.10)^{c}

Mean values and standard deviation in brackets.

$^{abc\ldots}$ different superscripts in the same column indicate statistically significant differences ($p < 0.05$)

Statistical analysis of the results showed, with a 95% confidence level, a significant effect of both the processing variables (the addition of 10% of trehalose by weight and the homogenization at 50 or 100 MPa) and the interaction between them. In general terms, the addition of 10 g of trehalose per 100 g of clementine juice caused a reduction in the apple rings volume (negative values of the impregnating parameter γ), but this slightly increased after the vacuum impregnation with free from trehalose liquids. This result seems logical considering that trehalose increases the solution hypertonicity, so that osmotic dehydration mechanisms appear coupled to the hydrodynamic ones due to the pressure gradients imposed to the system. For this reason, in addition to the possibly higher viscosity of the impregnating solutions, both the volume of apple that was filled with the impregnating liquid (X) and the solid matrix effective porosity (ε) noticeably decreased as a result of the addition of trehalose. However, due to the significant reduction in the average size of the particles present in the impregnating solution, these two impregnating parameters increased significantly after the homogenization at either 50 or 100 MPa.

3.5. Effect of processing variables on *Lactobacillus salivarius* spp. salivarius resistance to *in vitro* digestion

Probiotic survival during each step of the *in vitro* digestion (the gastric stage in acid media and the intestinal stage in basic media), as well as during the entire digestion process, is shown in Table 4. Values obtained after the whole gastrointestinal simulation were of the same order as those reported for other lactic acid bacteria, such as *Lactobacillus delbrueckii* spp. *bulgaricus* and *Streptococcus thermophilus* inoculated in a lactic substrate, with mean cumulative viabilities around 26.2% and 9.2%, respectively (García-Hernández et al., 2012), or *Lactobacillus reuteri* inoculated in raw and fried tomato, with mean cumulative viabilities around 24% and 26.3%, respectively (García-Hernández et al., 2018).

Table 4. *Lactobacillus salivarius* spp. *salivarius* CECT 4063 survival during the simulated gastrointestinal digestion as affected by the trehalose concentration and/or the homogenization pressure.

%TREH	P (MPa)	$\%VIAB_{ST1}$	$\%VIAB_{ST2}$	$\%VIAB_{ST1+ST2}$
0	0	23.3 (1.1)^{a}	121 (3)^{e}	28.1 (0.7)^{c}
	50	39.85 (1.04)^{d}	122 (4)^{e}	50 (4)^{e}
	100	33.3 (1.2)^{c}	109 (3)^{d}	36.5 (0.9)^{d}
10	0	51.3 (0.9)^{e}	59 (3)^{b}	30.2 (1.4)^{c}
	50	29.1 (0.6)^{b}	37 (2)^{a}	10.6 (0.4)^{a}
	100	24.3 (0.4)^{a}	87 (3)^{c}	21.3 (0.7)^{b}

Mean values and standard deviation in brackets.
$^{abc...}$ different superscripts in the same column indicate statistically significant differences ($p < 0.05$)

As expected, *Lactobacillus salivarius* spp. *salivarius* CECT 4063 was found to be more resistant to the basic pancreatin solution emulating bowel conditions than to the acid pepsin solution emulating gastric juices secreted in the stomach ($\%VIAB_{ST2} > \%VIAB_{ST1}$). Since the microbial counts were observed to slightly decrease along both the gastric and the intestinal step (Fig. 3), the probiotic bacteria difficulties in adapting to extreme acidic conditions at the beginning of the digestion process were assumed to be the main responsible for their loss of viability. However, when the probiotic bacteria were already stressed by the addition of 10% (w/w) of trehalose to the juice formulation, also passing from an acidic to a basic media resulted in a considerable decreased in the *Lactobacillus salivarius* spp. *salivarius* viability. This is why, although the only addition of 10 % (w/w) of trehalose to the juice doubled the probiotic survival to the gastric step, its survival to the whole gastrointestinal simulation was not significantly improved (p-value < 0.05).

As for the sublethal homogenization, its effect on the probiotic viability was dependent on both the addition of trehalose to the juice and the level of pressure applied. Regardless of the pressure applied, homogenization was observed to significantly increase the probiotic survival to both the gastric step and the whole digestion process (p-value < 0.05) in the juice containing no trehalose, but to significantly reduce those values in the juice containing 10% (w/w) of trehalose (p-value < 0.05). Regardless of the trehalose concentration, increasing from 50 to 100 MPa the homogenization pressure was reported to significantly reduce the percentage of cells remaining alive at the end of the gastric step. However, due to its different effect on the probiotic viability to the intestinal step, increasing from 50 to 100 MPa the homogenization pressure significantly reduced the probiotic survival to the whole gastrointestinal digestion in the juice containing no trehalose, but significantly increased that of the juice containing 10% (w/w) of trehalose. As a result of all this, the survival of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 to the *in vitro* digestion was maximum after homogenizing at 50 MPa the juice containing no trehalose but reached a minimum value after homogenizing at 50 MPa the juice containing 10% (w/w) of trehalose.

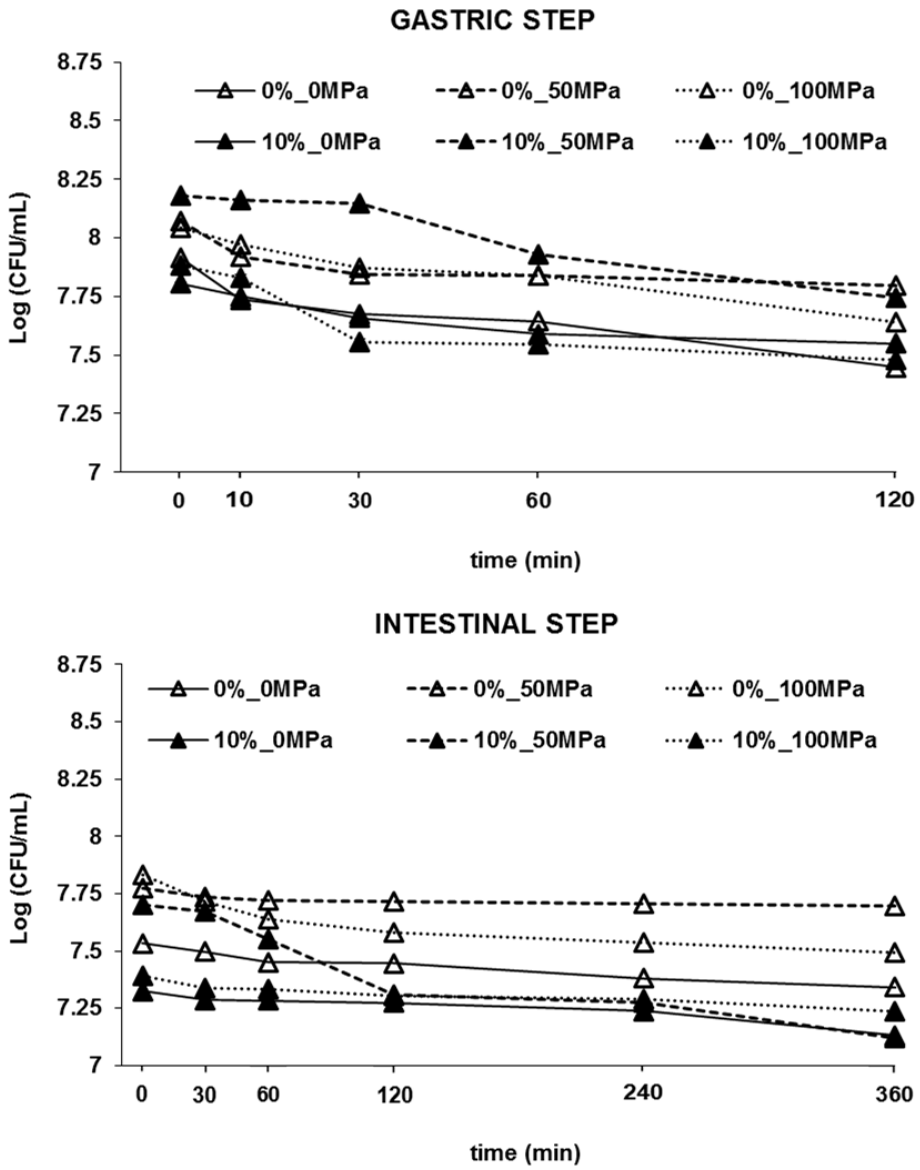


Figure 3. Survival of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 to the *in vitro* gastrointestinal digestion as affected by trehalose concentration.

In all cases, as it is evidenced in Fig. 3, the probiotic counts at the end of the digestion process resulted higher than 10^7 CFU/mL so the daily intake of 100 mL (less than half a glass) of any of these clementine juice-based liquids would provide the minimum dose required to provide a health benefit to the consumer (Collado et al., 2005; Sanders, 2008).

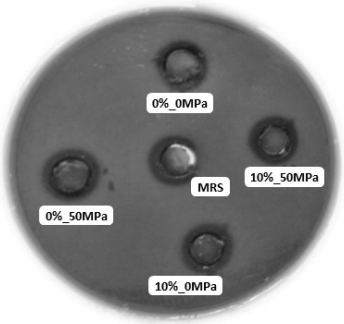
3.6. Effect of processing variables on anti-*Helicobacter pylori* effect of *Lactobacillus salivarius* spp. *salivarius*

Mean diameters of *Helicobacter pylori* growth inhibition by *Lactobacillus salivarius* spp. *salivarius* CECT 4063 grown in different media are shown in Table 5. According to the results obtained from *in vitro* digestion assays, authors decided to only evaluate anti-

Helicobacter pylori activity as affected by the addition of 10% (w/w) of trehalose to the juice before its inoculation and/or the juice homogenization at 50 MPa after incubation.

Table 5. Inhibition of *Helicobacter pylori* by *Lactobacillus salivarius* spp. *salivarius* strain CECT 4063 as affected by the growing media.

%TREH	P (MPa)	Ø inhibition (mm)
0	**0**	17 (2)[a]
	50	17.7 (0.6)[a]
10	**0**	17 (2)[a]
	50	16.7 (0.6)[a]
MRS		16.7 (0.6)[a]



Mean values and standard deviation in brackets.
[abc...] different superscripts in the same column indicate statistically significant differences ($p < 0.05$)

Results showed that *Lactobacillus salivarius* spp. *salivarius* strain CECT 4063 possesses the antagonistic activity to inhibit the growth of *Helicobacter pylori*, which was not significantly affected by the different growing media assessed. The validity of *Lactobacillus salivarius* as a probiotic to suppress *Helicobacter pylori* and thus reduce the inflammatory response was previously observed by Aiba et al. (1998). If as reported by Lin et al. (2011), anti-H*elicobacter pylori* activity is closely correlated with the concentration of organic acids and the pH value, those juices reaching a lower pH after the incubation with the probiotic (juices without trehalose) should exhibit a higher bactericidal activity against Helicobacter pylori. However, since clementine juice is naturally rich in flavonoids (hesperidin, narirutin, didymin, etc.) with antibacterial activity, differences in anti-*Helicobacter pylori* activity among probiotic beverages are minimal. In the particular case of MRS broth, the lack of flavonoids might be compensated with the higher microbial growth under optimal conditions.

4. Conclusions

Both physicochemical and microbial properties of clementine juice inoculated with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 were significantly affected by the addition of 10 or 20% of trehalose by weight to the juice formulation and/or the homogenization at 25, 50, 100 or 150 MPa. On one hand, increasing the trehalose concentration in the juice was observed to promote osmotic stress conditions and to significantly reduce the microbial counts, while notably increasing the probiotic resistance to both further homogenization and the shock produced by gastric juices during *in vitro* digestion. On the other hand, homogenization pressures below 150 MPa were reported to enhance (≤ 50 MPa) or maintain the amount of viable cells in the juice and to significantly increase the probiotic survival after both the gastric step and the whole gastrointestinal simulated process. Consequently, homogenizing at pressures not

exceeding 100 MPa the juice containing no trehalose managed to significantly increase the amount of living cells after *in vitro* digestion with proven ability to inhibit the *Helicobacter pylori* growth.

Regarding antioxidant properties of the samples, both total phenols and flavonoids content, together with the antioxidant activity, were mainly improved by the growth of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 in the juice. Moreover, the expected increase in total phenols bioavailability due to the decrease in the average size of the particles after the homogenization step was only evident when 10% of trehalose by weight was added to the juice formulation. On the contrary, due to their lower average particle size, homogenized juices showed higher ability to be used as impregnating liquids. In summary, the two factors considered in the present study might be combined in an appropriate way in order to improve the health benefits provided by the consumption of clementine juice inoculated with *Lactobacillus salivarius* spp. *salivarius* CECT 4063.

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ARTÍCULO 2

FERMENTATION OF CLEMENTINE JUICE WITH *LACTOBACILLUS SALIVARIUS* SPP. *SALIVARIUS* CECT 4063: EFFECT OF TREHALOSE ADDITION AND HIGH-PRESSURE HOMOGENIZATION ON ANTIOXIDANT PROPERTIES, MUCIN ADHESION, AND SHELF LIFE

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Abstract

Fermentation of fruit juices with lactic acid bacteria enhances their antioxidant properties to a different extent depending on the microbial strain and the growing media composition, which can be modified by adding certain ingredients or applying a homogenization step. This study analysed the effect of trehalose addition (10%, w/w) and homogenization at 100 MPa before or after *Lactobacillus salivarius* spp. *salivarius* CECT 4063 inoculation on the antioxidant profile and the microbiological properties of commercial clementine juice during 96 h fermentation. Antioxidant properties and viable cell count of 24 h-fermented juices during refrigerated storage (30 days at 4 °C) were also evaluated. Fermentation over 24 h reduced the microbial population and antioxidant content of clementine juice. Homogenizing the juice before inoculation enhanced the microbial growth but favoured antioxidant degradation. Adding trehalose (10%, w/w) to the juice formulation and/or homogenizing at the fermented juice at 100 MPa for 24 h had a negative impact on viable counts and did not improve the microbial adhesion to intestinal mucosa. However, both techniques prevented antioxidant oxidation and cell decay during the storage of fermented juice under refrigeration, which should not last more than 15 days.

Keywords: fermentation; *Lactobacillus salivarius* spp. *salivarius* CECT 4063; clementine juice; mucin adhesion; antioxidant properties; trehalose; high-pressure homogenization.

1. Introduction

Consumers are increasingly aware of the benefits of a healthy lifestyle. This is evident in the growing demand for functional foods that promote the maintenance and improvement of health, such as probiotic foods [1]. Probiotics are defined as live microorganisms intended to provide health benefits when administered in adequate amounts [2]. For these microorganisms to be included in foods, they must be generally recognized as safe (GRAS) as well as fulfil other requirements, including resistance to acidic conditions of the stomach and the digestive enzymes of the tract, adherence to intestinal mucosa, competitive exclusion of pathogens, survival from industrial production and storage conditions, safety, and scientifically proven efficacy. Benefits derived from probiotic consumption include the prevention of diarrhea caused by pathogenic bacteria and viruses, the stimulation of the host's immune system, the reduction of the symptoms of lactose intolerance, and the prevention of constipation,

among others [3,4]. *In vitro* and *in vivo* studies show that some lactic acid bacteria are also able to inhibit the growth of *Helicobacter pylori* and reduce the activity of the enzyme urease, required by the pathogen to resist the acidic conditions of the stomach [4]. This group includes several *Lactobacillus salivarius* strains, such as *L. salivarius strains B37* and *B60* [5], *L. salivarius* spp. *salicinius AP-32* [6] and *L. salivarius* spp. *salivarius* CECT 4063 [7].

In addition to these benefits, biochemical changes taking place during fermentation with probiotics also contribute to enhance nutritional properties of certain foods. Fermentation of fruit juices with lactic acid bacteria has been shown to convert certain polyphenols into other metabolites with higher antioxidant activity and/or higher bioavailability [8]. In soy-derived products, fermentation with LAB improved protein and mineral bioavailability by reducing antinutritional factors, such as phytic acid [9].

As regards the viability of probiotics in food products, it depends on several factors [10]: (a) factors related to the composition of the food matrix, such as pH, salt and sugar content or oxygen concentration; (b) factors related to the microorganism, such as the specific strain or the inoculum concentration; and (c) factors related to the processing and storage conditions. In the manufacture of a probiotic snack by means of vacuum impregnation and further dehydration [11], the higher the microbial concentration in the impregnation liquid, the greater the counts in the vacuum-impregnated product. Then, the decrease in the microbial population undergone by the impregnated product during its further processing can be reduced in different ways. Techniques employed to extend the shelf life of probiotics in foods from production to consumption include the modification of processing and storage conditions, the addition of different protective agents, or encapsulation [10]. Among protective agents, trehalose is a nonreducing disaccharide made up of two glucose molecules that acts as a reserve sugar and confers protection against abiotic stress in various organisms [12]. In dehydration processes, trehalose favours the fluid state of lipids, thus preventing their fusion, phase separation and the rupture of biological membranes. In addition, the liquid-to-gel transition is delayed by replacing water molecules with trehalose molecules [13].

The functionality of probiotics can also be enhanced by means of high-pressure homogenization (HPH). This is a nonthermal technology that is usually applied to liquid foods to inactivate enzymes, decrease the microbial load, improve their sensory and technological quality, and increase the availability of active compounds [14]. On probiotic microorganisms, HPH at sublethal pressures has been proven to be effective in improving hydrophobicity, self-aggregation, and resistance to biological stress in different food matrices, in addition to preserving their viability during storage in refrigeration [15,16]. Cellular hydrophobicity is directly related to the adhesion capacity of the microbial strain and as previously mentioned, its interaction capacity with the gut. Resistance to harsh conditions induced by HPH includes the probiotic passage through the gastrointestinal tract [7].

The objective of the present study was to evaluate the effect of trehalose addition (10% w/w) and homogenization (100 MPa) before or after *Lactobacillus salivarius* spp. *salivarius* CECT 4063 inoculation on the microbial growth and antioxidant profile of commercial clementine juice during 96 h fermentation. Mucin adhesion, antioxidant properties and viable cell counts of 24 h fermented juices during refrigerated storage

(30 days 4 °C) were also evaluated. The purpose was to obtain a liquid with maximum microbial and antioxidant contents to be used as an impregnating liquid in vacuum-impregnation processes.

2. Materials and Methods

2.1. Raw Materials and Microbial Strain

Pasteurized and refrigerated squeezed clementine juice was purchased from a local supermarket (Valencia, Spain) and kept refrigerated at 4 °C in its own airtight Tetra Brik® container until use. Food-grade trehalose from tapioca starch (TREHA™) was supplied by Cargill Inc. (Barcelona, Spain).

Lactobacillus salivarius spp. *salivarius* CECT 4063 from the Spanish Type Culture Collection (University of Valencia, Burjassot, Spain) was used in this study. The strain was cultured in MRS broth (Scharlau Chemie®, Barcelona, Spain) at 37 °C. After 24 h in aerobiosis, broths with a microbial concentration of 10^9 CFU/mL measured by plate count were obtained.

2.2. Juice Conditioning, Fermentation, and Storage

As described by Barrera et al. [7], 5 g/L of yeast extract (Panreac Química S.L.U., Barcelona, Spain) were added to commercial clementine juice together with 9.8 g/L of food-grade sodium bicarbonate so that its pH was raised from 3.8 to 6. In samples containing trehalose, 10% (w/w) of such disaccharide was incorporated into the juice formulation. Once the ingredients were dissolved, part of the liquid was homogenized at 100 MPa in a laboratory-scale high-pressure homogenizer (Panda Plus 2000, GEA-Niro Soavi, Parma, Italy). Then, both homogenized (HPH) and unhomogenized (non-HPH) juices were inoculated with 4mL/L of MRS broth containing the activated microorganism and incubated at 37 °C in aerobiosis for 96 h. After 24 h of incubation, part of the unhomogenized sample was homogenized at 100 MPa and returned to incubation to complete the 96 h of fermentation (I+HPH). Fermented samples were collected at 0, 24 (before and after homogenizing), 48, 72, and 96 h for microbiological and chemical analysis. Fermenting for such long periods will not only provide information about the effect of the processing variables on the microbial growth during the log phase but also on the exponential de-crease in the number of living cells during the death phase. Non-HPH and I+HPH liquids (with and without 10% (w/w) of trehalose) that were fermented for 24 h underwent the storage study at 4 °C for 30 days. Sampling in this case took place at days 0, 1, 2, 3, 7, 10, 15, 21, and 30.

2.3. Microbial Counts

Colony counts of live *Lactobacillus salivarius* spp. *salivarius* CECT 4063 present in each juice were conducted throughout fermentation and storage by serial dilution in sterile peptone water and plating on the surface of MRS agar (Scharlau Chemie®, Barcelona, Spain) plates. Then, the plates were incubated at 37 °C for 24 h under aerobic conditions.

2.4. pH and Brix

Brix values were obtained from the refractive index of the juices measured at 20 °C in an optical refractometer (Abbe NAR-T3, ATAGOTM, Tokyo, Japan). The pH of the

different liquids was measured at 25 °C with a benchtop pH meter (S20 Seven Compact EasyTM, Mettler Toledo, Barcelona, Spain) correctly calibrated with buffer solutions at pH 4.0 and 7.0.

2.5. Antioxidant Properties

2.5.1. Total Phenolic Content (TPC)

Total phenolic content was determined by the Folin-Ciocalteu method [17], based on the ability of phenolic compounds to reduce a mixture of phosphotunstic and phosphomolybdic acids in a basic medium to form blue tungsten and molybdenum oxides. To carry out this analysis, 125 µL of the sample diluted in bidistilled water in a 1:5 (v/v) ratio was mixed with 125 µL of the Folin-Ciocalteu reagent and 500 µL of bidistilled water. After 6 min in darkness, 1.25 mL of sodium bicarbonate at 7.5% (w/v) and 1 mL of bidistilled water were added. A blank in which the sample was replaced with distilled water was used as a reference. After 90 min in darkness, the absorbance was measured at 760 nm in a Helios Zeta UV/vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Results were expressed in mg of gallic acid equivalents per mL of juice (mg GAE/mL).

2.5.2. Total Flavonoid Content (TFC)

The total flavonoid content (TFC) was determined by the colorimetric method of aluminium chloride described by Luximon-Ramma et al. [18]. Briefly, 1.5 mL of the sample diluted in bidistilled water in a 1:5 (v/v) ratio was mixed with 1.5 mL of aluminium chloride in methanol at 2% (w/v). A blank in which the sample was replaced with distilled water was used as a reference. After 10 min in darkness, the absorbance was measured at 368 nm in a Helios Zeta UV/vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Results were expressed in mg of quercetin equivalents per mL of juice (mg QE/mL).

2.5.3. Antioxidant Activity (AO)

Total antioxidant capacity of juice samples was measured by both ABTS-TEAC and DPPH assays. The ABTS-TEAC assay is based on measuring the discoloration undergone by the radical 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) when reduced in the presence of oxygen-donor antioxidants [19]. For the ABTS-TEAC assay, 90 µL of each sample diluted in bidistilled water in a 1:5 (v/v) ratio was added into 2910 µL of a 7 mM ABTS+ solution in phosphate buffer (A734 nm = 0.7 ± 0.01). A white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. After 6 min of reaction in the dark, the absorbance was measured at 734 nm in a Helios Zeta UV/vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Results were expressed in mg of Trolox equivalents per mL of juice (mg TE/mL).

DPPH assay is based on measuring the discoloration undergone by the radical 2,2-diphenyl-1-picrylhydrazyl due to the donation of hydrogen atoms in the presence of antioxidant compounds [20]. To carry out this analysis, 100 µL of the sample diluted in bidistilled water in a 1:5 (v/v) ratio was added into 2000 µL of a 100 mM solution of $DPPH^{\bullet}$ in methanol and 900 µL of methanol (purity ≥ 99%). A white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. After 30 min of reaction in the dark, the absorbance was measured at 517 nm in

a Helios UV/vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Results were ex-pressed in mg of Trolox equivalents per mL of juice (mg TE/mL).

2.5.4. Quantification of Individual Flavonoids by HPLC

Extraction and analysis of the major flavonoids, i.e., narirutin (NAR), hesperidin (HESP) and didymin (DID), was adapted from Cano et al. [21]. Aliquots of 6 mL of juice were mixed with 4 mL of a 1:1 (v/v) solution of dimethyl sulfoxide in methanol. After stir-ring for 1 min at 1000 rpm in a GFL compact orbital shaker 3005, the mixture was centrifuged at 4 °C for 30 min at 10,000 rpm (Medifriger BL-S, J.P. Selecta, Barcelona, Spain). The collected supernatant was filtered through a 0.45 µm nylon filter and directly injected into a C18 reversed-phase column (250 × 4.6 mm and 5 µm) installed in a Waters Alliance 2695 HPLC (Waters Inc., Milford, CT, USA) equipped with a photo diode array detector. A mobile phase consisting of acetonitrile (solvent A) and 0.6% (v/v) acetic acid (solvent B) was used for analysis. The test conditions were as follows: 10% of solvent A for 2 min, increase the concentration of solvent A progressively until reaching 50% during the following 18 min, hold for 5 min, and return to the initial conditions in 5 min. A constant flow rate of 1 mL/min was used. Compounds were identified comparing their retention times (13.7 min, 14.3 min and 17.2 min for NAR, HESP and DID, respectively) and UV-vis spectra at 280 nm with the corresponding standards. Concentrations were obtained using an external standard curve.

2.6. Adhesion Assay

Based on the method described by Izquierdo et al. [22], *Lactobacillus salivarius* spp. *salivarius* CECT 4063 cells from 24-hour-old cultures grown in MRS broth or conditioned clementine juice were collected by centrifugation (4000 rpm for 10 min at 4 °C), washed twice, and resuspended in phosphate buffered saline (PBS) solution. Next, the cell suspension absorbance measured at 600 nm was adjusted to 0.25 ± 0.05, equivalent to a microbial con-centration of 10^7-10^8 CFU/mL. Then, cell suspensions were labelled in the dark with 75 µm carboxyfluorescein diacetate (Sigma Aldrich, Steinheim, Germany) for 1 h at room temperature. Finally, microbial cells were washed twice and resuspended in PBS.

In parallel, 500 ppm solutions in PBS were prepared for each of the three different proteins assayed: mucin from porcine stomach (MUC), collagen from calf skin (COL) and bovine serum albumin (BSA), all purchased from Sigma-Aldrich (Steinheim, Germany). Then, 100 µL of each solution was loaded into black polystyrene 96-well cell culture plates (Thermo Fisher Scientific, Nunc Edge 2.0, Waltham, MA, USA) and incubated overnight at 4 °C. To remove unadhered proteins, the wells were washed three times with 200 µL PBS. Next, 100 µL of working labelled bacterial suspension was added per well. After incubating the plates for 1 h at 37 °C, the wells were washed twice with 200 µL PBS to remove unbound bacteria and 100 µL of PBS were added to each well containing adhered bacteria. The released fluorescence was red in a FluoroskanTM microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 485 nm excitation and 538 nm emission. Adhesion was expressed as the percentage of fluorescence recovered after binding relative to the fluorescence of the bacterial suspension initially added to the wells.

2.7. Statistical Analysis

The statistical significance of the results was evaluated with the Statgraphics Centurion XVI tool, by means of simple and multivariate analysis of variance with a 95% confidence level.

3. Results

3.1. Fermentation of clementine juice by *Lactobacillus salivarius* spp. *salivarius* CECT 4063 as affected by trehalose addition and/or juice high pressures homogenization.

Figure 1 shows the effect of the homogenization treatment applied and the addition of trehalose (10%, w/w) on the *Lactobacillus salivarius* spp. *salivarius* CECT 4063 content of conditioned clementine juice throughout the fermentation process. As it can be observed, the variation of cell counts over incubation time resembled that of the theoretical variation showing the microbial growth under limited conditions of volume and nutrients content. In all cases, maximum growth occurred after 24 h of fermentation, which meant a slight decrease in Brix (from 19.3 ± 0.2 to 18.6 ± 0.4 and from 12.8 ± 0.5 to 12.4 ± 0.6 for juices formulated with and without trehalose, respectively) and a notable decrease in pH (from 6.54 ± 0.12 to 4.368 ± 0.012 for juices formulated with and without trehalose) due to lactic acid production; this led to the cell death phase, in which both Brix and pH remained almost constant.

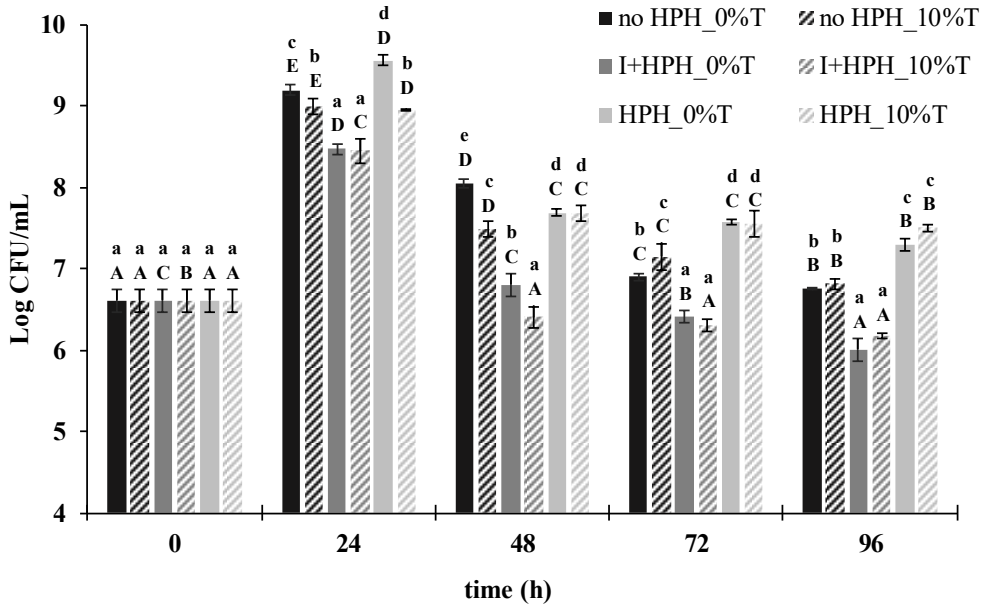

Figure 1. Microbial counts in mandarin juice subjected to different treatments along their fermentation with *L. salivarius* spp. *salivarius* (CECT 4063) for 4 days. Error bars represent the standard deviation of 4 replications. $^{a-c}$ in the same time and $^{A-D}$ in the same series indicate statistically significant difference with a 95% confidence level ($p < 0.05$).

As shown in Table 1, the increase in the microbial population was significantly enhanced ($p < 0.05$) by the homogenization of the juice at 100 MPa before its inoculation

(treatment HPH_0%T). It follows from this that considerable reduction in the pulp particle size reported for HPH treatments applied to citrus juices as a result of pectin depolymerization might not only increase the juice cloudiness and reduce the juice viscosity [23,24] but also notably increase precocious availability of some chemical components that are essential for the lactobacillus strain growth. In milk for cheesemaking, as an example, significant increase in the availability of low-molecular-weight peptides and free fatty acids such as oleic acid was observed by Burns et al. [25] after homogenization at 100 MPa. In orange juice, decrement of the size of the cloud fraction after homogenization at 200 and 300 MPa significantly improved the extractability of flavonoids and increased the amount of soluble flavanones [26]. Conversely, as a preservation technique, the homogenization of the juice that had been previously inoculated and incubated for 24 h at 37 °C caused a significant microbial inactivation, as deduced from the lowest log increase values reached by the I+HPH juices in this interval. In the same way, cell exposure to osmotic stress resulting from trehalose addition to the growing media and the subsequent Brix increase from 12.8 ± 0.4 to 19.3 ± 0.4 had a negative impact on the growth and multiplication of bacteria. In any case, the microbial content of the juices after 24 h of fermentation ranged between (2.9 ± 0.4) × 10^8 and (3.69 ± 0.6) × 10^9 CFU/mL, which is higher than the minimum concentration of 10^6-10^7 CFU/mL required to make an EU-based health claim [27].

Table 1. Log increase (values > 0) or log decrease (values < 0) respectively showing the *L. salivarius* spp. *salivarius* growth or demise from the juices along their fermentation. Mean value of 4 replications ± standard deviation. $^{a-c}$ in the same interval and $^{A-D}$ in the same treatment indicate statistically significant difference with a 95% confidence level (p < 0.05).

treatment		Interval			
		0-24h	24-48h	24-72h	24-96h
no HPH	0%T	2.54 ± 0.06^{dD}	-1.13 ± 0.05^{dC}	-2.26 ± 0.05^{aB}	-2.400 ± 0.014^{abA}
	10%T	2.33 ± 0.10^{cD}	-1.48 ± 0.10^{cC}	-1.81 ± 0.16^{bB}	-2.13 ± 0.06^{cA}
I+HPH	0%T	1.70 ± 0.07^{aD}	-1.70 ± 0.14^{bC}	-2.11 ± 0.08^{aB}	-2.51 ± 0.14^{aA}
	10%T	1.68 ± 0.15^{aC}	-2.08 ± 0.13^{aB}	-2.18 ± 0.07^{aAB}	-2.32 ± 0.03^{bA}
HPH	0%T	2.68 ± 0.07^{eC}	-1.70 ± 0.04^{bB}	-1.80 ± 0.03^{bB}	-2.05 ± 0.08^{cA}
	10%T	2.153 ± 0.010^{bC}	-1.16 ± 0.09^{dB}	-1.3 ± 0.2^{cB}	-1.52 ± 0.04^{dA}

As regards the decrease in the microbial population observed after 24 h of fermentation, it was initially very abrupt for treatments HPH and I+HPH (interval 24-48 h), but in the end (interval 24-96 h) it was significantly less evident for treatment HPH. This suggests that *Lactobacillus salivarius* spp. *salivarius* CECT 4063 survival in adverse conditions, basically due to nutrient depletion, low pH, and the accumulation of organic acids with antimicrobial activity resulting from cellular metabolism, was not in this case enhanced by its sublethal homogenization. Instead, the homogenization of the juice prior to inoculation seemed to reinforce the ability of some food components to protect cells from acidic stress and/or that of antioxidant compounds to prevent oxidative stress [28]. In addition, the addition of 10% (w/w) of trehalose to the juice prevented in all cases the lactobacillus strain from decay after 24 h of fermentation, just as previously observed during the freeze-drying of vacuum-impregnated apple slices and after 30-day storage

of apple snacks that had been dried with air at 40 °C for 48 h [29] or at the end of the gastric step in the course of in vitro digestion of clementine juice inoculated with *Lactobacillus salivarius* CECT 4063 [7]. Consequently, the minimal microbial count (1.0 ± 0.3) × 10^6 cfu/mL) was achieved after fermenting for 96 h the clementine juice that was first inoculated and incubated for 24 h at 37 °C and then homogenized at 100 MPa.

Table 2. Antioxidant properties of clementine juice subjected to different treatments along their fermentation with *L. salivarius* spp. *salivarius* CECT 4063 for 4 days.

		t (h)	Total Phenols (mg GAE/mL)	Total Flavonoids (mg QE/mL)	AO_DPPH (mg TE/mL)	AO_ABTS (mg TE/mL)
no HPH	0%T	**0**	0.84 ± 0.08 ^{aAB}	0.747 ± 0.014 ^{aA}	0.33 ± 0.02 ^{aA}	1.35 ± 0.06 ^{dD}
		24	1.81 ± 0.13 ^{dC}	2.09 ± 0.03 ^{bcD}	0.51 ± 0.04 ^{cB}	1.61 ± 0.08 ^{cE}
		48	0.99 ± 0.09 ^{bB}	1.65 ± 0.07 ^{cC}	0.31 ± 0.04 ^{abA}	0.92 ± 0.05 ^{bC}
		72	0.86 ± 0.06 ^{abAB}	1.64 ± 0.07 ^{cC}	0.32 ± 0.03 ^{cA}	0.85 ± 0.04 ^{bB}
		96	0.75 ± 0.09 ^{aA}	1.130 ± 0.002 ^{bB}	0.309 ± 0.012 ^{cA}	0.69 ± 0.07 ^{bA}
	10%T	**0**	0.90 ± 0.05 ^{bB}	0.89 ± 0.04 ^{bA}	0.31 ± 0.03 ^{aAB}	1.34 ± 0.09 ^{cdC}
		24	2.00 ± 0.10 ^{eC}	2.19 ± 0.11 ^{cD}	0.49 ± 0.02 ^{bcC}	1.53 ± 0.02 ^{cD}
		48	0.79 ± 0.03 ^{aAB}	1.4 ± 0.2 ^{aC}	0.34 ± 0.05 ^{bB}	0.78 ± 0.03 ^{aB}
		72	0.78 ± 0.03 ^{aA}	1.15 ± 0.06 ^{aB}	0.310 ± 0.011 ^{cAB}	0.719 ± 0.014 ^{aA}
		96	0.7 ± 0.2 ^{aA}	1.10 ± 0.03 ^{aB}	0.28 ± 0.03 ^{bA}	0.678 ± 0.005 ^{abA}
l + HPH	0%T	**0**	0.81 ± 0.04 ^{aA}	0.747 ± 0.014 ^{aA}	0.33 ± 0.02 ^{aD}	1.26 ± 0.04 ^{bcC}
		24	1.5 ± 0.2 ^{cD}	1.99 ± 0.09 ^{bC}	0.37 ± 0.03 ^{aE}	1.35 ± 0.06 ^{bD}
		48	1.37 ± 0.13 ^{cCD}	2.00 ± 0.05 ^{dC}	0.28 ± 0.03 ^{aC}	1.23 ± 0.05 ^{dC}
		72	1.26 ± 0.04 ^{cBC}	1.87 ± 0.14 ^{dBC}	0.18 ± 0.02 ^{aB}	1.11 ± 0.07 ^{cB}
		96	1.18 ± 0.05 ^{cB}	1.8 ± 0.2 ^{dB}	0.116 ± 0.006 ^{aA}	0.76 ± 0.07 ^{bA}
	10%T	**0**	0.90 ± 0.05 ^{bA}	0.89 ± 0.04 ^{bA}	0.31 ± 0.03 ^{aC}	1.19 ± 0.08 ^{bB}
		24	1.03 ± 0.14 ^{bB}	1.65 ± 0.02 ^{aD}	0.46 ± 0.03 ^{bD}	1.38 ± 0.13 ^{bC}
		48	0.97 ± 0.07 ^{bAB}	1.56 ± 0.06 ^{bcC}	0.29 ± 0.02 ^{aC}	1.14 ± 0.02 ^{cB}
		72	0.96 ± 0.09 ^{bAB}	1.55 ± 0.03 ^{bBC}	0.214 ± 0.008 ^{bB}	1.1 ± 0.2 ^{cB}
		96	0.94 ± 0.05 ^{bAB}	1.50 ± 0.05 ^{cB}	0.119 ± 0.003 ^{aA}	0.69 ± 0.03 ^{bA}
HPH	0%T	**0**	0.84 ± 0.04 ^{aA}	0.99 ± 0.02 ^{dA}	0.30 ± 0.01 ^{aA}	0.83 ± 0.04 ^{aC}
		24	0.83 ± 0.03 ^{aA}	1.58 ± 0.10 ^{aC}	0.51 ± 0.07 ^{cD}	0.88 ± 0.03 ^{aC}
		48	0.80 ± 0.06 ^{aA}	1.44 ± 0.12 ^{abB}	0.47 ± 0.03 ^{cCD}	0.82 ± 0.11 ^{aC}
		72	0.81 ± 0.06 ^{aA}	1.44 ± 0.09 ^{bB}	0.41 ± 0.03 ^{dBC}	0.69 ± 0.08 ^{aB}
		96	0.75 ± 0.07 ^{aA}	1.06 ± 0.06 ^{aA}	0.39 ± 0.06 ^{dB}	0.59 ± 0.10 ^{aA}
	10%T	**0**	0.80 ± 0.04 ^{aC}	0.94 ± 0.04 ^{cA}	0.33 ± 0.03 ^{aA}	0.80 ± 0.06 ^{aB}
		24	0.82 ± 0.05 ^{aC}	1.6 ± 0.2 ^{aC}	0.50 ± 0.02 ^{cD}	0.88 ± 0.05 ^{aC}
		48	0.79 ± 0.08 ^{aBC}	1.53 ± 0.14 ^{bcC}	0.47± 0.04 ^{cC}	0.79 ± 0.03 ^{aAB}
		72	0.72 ± 0.05 ^{aA}	1.45 ± 0.06 ^{bC}	0.426 ± 0.014 ^{dB}	0.73 ± 0.07 ^{aAB}
		96	0.72 ± 0.05 ^{aAB}	1.20 ± 0.04 ^{aB}	0.359 ± 0.013 ^{dA}	0.72 ± 0.08 ^{bA}

For each column, $^{a-c}$ in the same time and $^{A-D}$ in the same treatment indicate statistically significant difference with a 95% confidence level ($p < 0.05$). Mean ± standard deviation of 4 replications.

As deduced from the results obtained at time 0 h (Table 2), homogenizing the juice at 100 MPa before its inoculation affected neither the TPC nor the ability to scavenge $DPPH^{\bullet}$ free radicals, but significantly increased and decreased the TFC and the ability to scavenge $ABTS^{+}$ free radicals, respectively. Regarding trehalose addition, it hardly affected the AO activity measured by the application of both $DPPH^{\bullet}$ and $ABTS^{+}$ free radicals, but significantly increased the TPC and TFC of unhomogenized juice samples. These results confirm the ability of such disaccharides to prevent the oxidation of certain

phenolic compounds reported by Kopjar et al. [30]. However, by homogenizing the juice and reducing the size of the pulp, certain antioxidants (such as ascorbic acid or vitamin C) might be more exposed to light and dissolved oxygen [31] and the amount of trehalose added to the juice formulation is not enough to prevent their rapid degradation. Probably for this reason, the AO activity measured by the ABTS method had significantly lower values in HPH juices. When analysed by the $DPPH^{\bullet}$ free radical method, the AO activity of the juices was much lower and was not affected by the process variables, which may be due to the lower affinity of this radical for hydrophilic compounds [32].

All the AO properties analysed improved within the first 24 h of fermentation and then progressively decreased (Table 2). Increase in TPC was previously observed in heat- and HHP-treated lychee juice after fermentation for 18 h by *Lactobacillus casei* at 30 °C due to large polymeric phenolics degradation by the microbial strain [31]. In addition, in blueberry juice, the TPC increased by 6.1–81.2% and the AO capacity in vitro was enhanced by at least 34% under fermentation at 37 °C for 48 h with autochthonous lactic ac-id bacteria due to derivation of phenolics among themselves, the metabolism of organic acids and other metabolic pathways [33]. In the present study, increase in TPC and TFC after fermentation of clementine juice for 24 h with *Lactobacillus salivarius* CECT 4063 ranged between 3.1 ± 0.7% and 123 ± 12% and between 56 ± 4% and 180 ± 4%, respectively.

Increase in TPC and TFC was minimum for HPH juices and maximum for non-HPH ones, which also underwent the highest microbial growth in the same interval. Small concentrations of dissolved oxygen in juices summited to high pressure treatments were reported in previous studies to accelerate the oxidation of ascorbic acid [31] and to cause the degradation of several nutraceutical compounds, including anthocyanins and poly-phenols, over time [34]. Contrary to what has been previously commented for unfermented samples, the homogenization at 100 MPa after lactic acid fermentation for 24 h reduced both TPC and TFC of the juice, especially when trehalose was added to its formulation. From this, it follows that the phenolic compounds resulting from the microbial activity were more sensitive to high homogenization pressures than those naturally present in the juice. Differences found in TPC and TFC increase depending on the treatment did not significantly affect the rise in the capacity to scavenge free radicals of clementine juice due to fermentation, which was calculated in percentage as the difference between the values obtained for 24 h fermented and unfermented liquids referred to the values obtained for unfermented liquids and were around 57 ± 8% and 11 ± 4% for $DPPH^{\bullet}$ and $ABTS^{+}$, respectively. Only the absence of trehalose seemed to slow down the increase in the AO activity measured by the $DPPH^{\bullet}$ free radical method in sample I+HPH, as deduced from the values obtained for unfermented and 24 h-fermented liquids, shown in Table 2.

To confirm these results obtained by spectrophotometric analysis techniques, the content of some of the most abundant flavonoids present in clementine juice were analysed by chromatographic techniques. Figure 2 shows the concentration of

hesperidin, narirutin, and didymin in each of the juices analysed before and after 24 h of fermentation at 37 °C with *Lactobacillus salivarius* spp. *salivarius* CECT 4063.

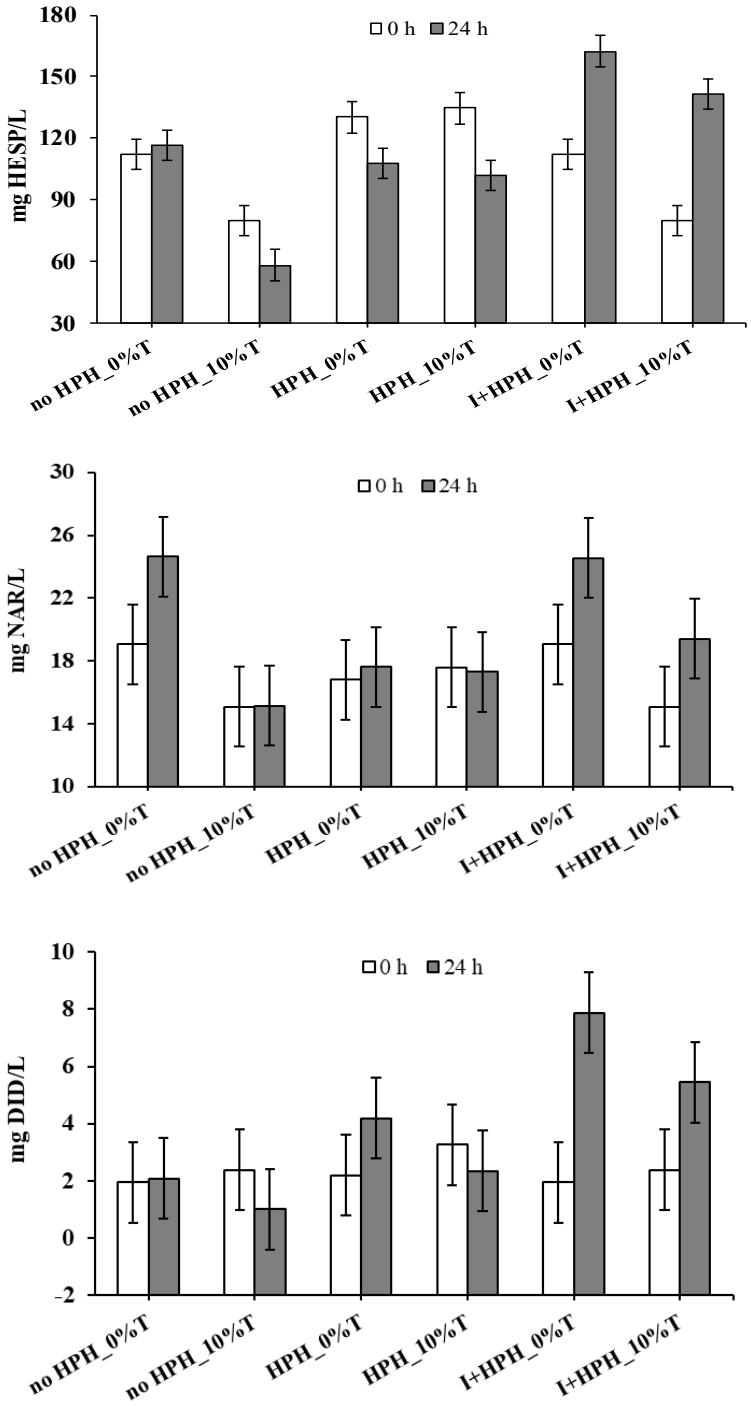

Figure 2. Hesperidin (HESP), narirutin (NAR) and didymin (DID) content in the juice samples before and after 24 h of fermentation at 37 °C. Graphs of means and LSD intervals for a confidence level of 95%.

Among the flavonoids analysed, hesperidin was found to be the most abundant in clementine juice, followed by narirutin and didymin. Values obtained were similar to those reported by Cano et al. [21] in a study on clementine pulp of different varieties, with a hesperidin content between 132 and 354 mg/kg and a narirutin content between 26 and 76 mg/kg. In a different study on fresh ortanique juice [35], hesperidin, narirutin and didymin contents were 116 ± 3 mg/L, 41.75 ± 0.02 mg/L and 14.3 ± 0.3 mg/L, respectively, and these values were not affected by the homogenization in the range between 5 and 30 MPa.

Focusing on freshly inoculated samples, only hesperidin was significantly ($p < 0.05$) more abundant in the juices that were homogenized at 100 MPa before its inoculation with the microbial strain (HPH samples). These samples were the ones that also presented the lowest AO activity measured by the ABTS+ free radical method. After fermentation for 24 h, the expected increase in each individual flavonoid content was only evident in those juices that were subsequently homogenized at 100 MPa (I+HPH samples). However, according to the spectrophotometric analysis technique, non-HPH juices were the ones with the highest content of total flavonoids after 24 h of fermentation. Possibly, the fact of using quercetin with maximum absorbance at 368 nm as a reference flavonoid to express the total flavonoid content is the cause of such discrepancy between the results obtained by the different analysis techniques used.

Oxidation reactions continued 24 h after the start of fermentation, which together with the loss of viability of the lactobacillus strain, caused a decline in all the AO properties of the juice. Degradation of AO compounds was notably less evident for HPH samples, in which the microbial decay was also observed to be minimum. On the contrary, the decrease in TPC, TFC and in the ability to scavenge the $ABTS^{+}$ free radical (calculated in percentage, as the difference between the values obtained for 96 fermented and 24 h fermented liquids refers to the values obtained for 24 h fermented liquids shown in Table 2) was maximum in non-HPH_0%T juice (59 ± 5%, 35 ± 12% and 43 ± 3%, respectively) and more or less slightly increased when adding 10% (w/w) of trehalose to its composition (66 ± 9%, 49.7 ± 1.4% and 55.8 ± 0.3%, respectively). In a different study, trehalose addition to orange jelly reduced from 27.8% to 9.14% the loss of phenols during storage [36]. In the present study, positive influence of trehalose on the phenol content was also observed for I+HPH samples. As a result of these changes in the content of antioxidant compounds, the ability to scavenge the $ABTS^{+}$ free radical after fermenting the juices for 96 h with *Lactobacillus salivarius* CECT 4063 was even lower than that of unfermented samples.

3.2. Adhesion to Intestinal Epithelium after Fermentation as Affected by Trehalose Addition and/or Juice Homogenization

The adhesion to intestinal epithelium is one of the most important characteristics for a potentially successful probiotic. It is related to the microbial ability to colonize mucosal surfaces, to interfere with pathogen binding and to interact with the immune system cells [37], and so it is crucial for a probiotic strain to exert a beneficial effect on the host. To get adhered to the intestinal mucosa, probiotics have specific adhesion proteins on

their surface that are recognized by the receptors of the epithelial cells in the small intestine. A specific interaction, such as hydrophobic interaction and aggregating interaction between surface macromolecules, are also involved in the colonization potential of probiotics and its contribution to the competitive exclusion of pathogens and to the improvement of the gut microbiome [38].

A critical first step in the development of most infections, bacterial adherence has become a target for the development of novel therapeutics and functional foods containing probiotics [39]. To assess the adhesive capacity of *Lactobacillus* to the intestinal mucosa, several *in vitro* model systems have been developed, including binding of bacteria to immobilized proteins and its quantitative measurement by fluorescent hybridization [40], as was used in this work. Figure 3 shows the capacity of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 strain to adhere to mucin (MUC), collagen (COL) and bovine serum albumin (BSA) as affected by the growing media (MRS broth or clementine juice formulated with and without 10% by weight of trehalose) and/or its homogenization at 100 MPa. Since they did not induce any stress in the microbial strain, HPH samples were not subjected to this analysis.

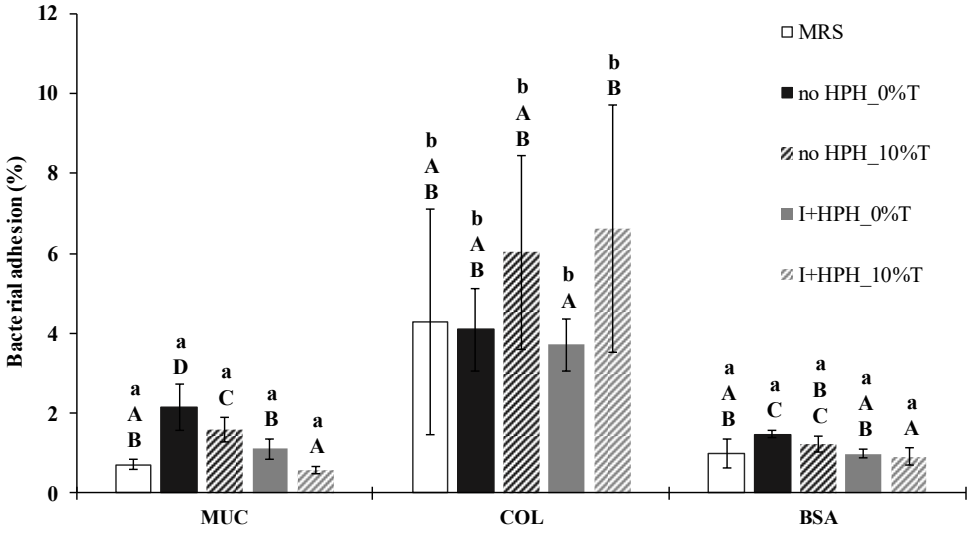

Figure 3. *Lactobacillus salivarius* spp. *salivarius* CECT 4063 adhesion to mucin (MUC), collagen (COL) and bovine serum albumin (BSA) depending on the growth media composition and/or the homogenization at 100 MPa. Error bars represent the standard deviation of 6 replications. $^{a-b}$ for the same growing media and $^{A-D}$ for the same protein indicate statistically significant differences with a 95% confidence level ($p < 0.05$).

As can be seen, the percentage of cells that adhered to collagen was in all cases higher than that adhered to mucin or bovine serum albumin. This makes sense, since 70% of *Lactobacillus* isolates have been reported to express multiple adhesin types interacting with the collagen of the intestinal mucosa, which plays a decisive role in the competitive dis-placement of intestinal pathogens such as *Escherichia coli* 0157:H7 [41]. *Lactobacillus salivarius* spp. *salivarius* CECT 4063 attachment to the collagen surface was slightly lower than that reported by Rokana et al. [42] for *L. bulgaricus* NCDC26 (8.36 ± 1.47%) and *L. casei* ATCC393 (7.85 ± 1.72%), but considerably higher than that observed

in *L. plantarum* NCDC20 (0.73 ± 0.52%) and *L. fermentum* NCDC214 (0.15 ± 0.03%). High deviations obtained for adhesion to COL values suggest that non-specific interactions (such as hydrophobic interactions and aggregating interactions) might play a more important role in this case. Since such nonspecific interactions are reported to be largely predisposed to the composition of bacterial surface covering components [41], slight modifications to the growing media composition or in the processing conditions might have a greater impact on them. Coupling between specific and non-specific interactions would also explain that the ability of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 to adhere to COL was in general terms higher than that to MUC.

Compared to MRS broth, the use of clementine juice (non-HPH_0%T) as growing medium increased the capacity of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 to adhere to any of the three proteins considered. This suggests that clementine juice is not only an excellent matrix for the growth of the microbial strain but also for the improvement of its probiotic properties. Neither the addition of trehalose to the juice before its inoculation (non-HPH_10%T), the homogenization at 100 MPa of the inoculated and 24 h fermented juice (I+HPH_0%T) nor the combination of the two factors (I+HPH_10%T) significantly improved the ability of the microorganism to adhere to the proteins tested.

As regards the trehalose addition to the growing medium, it was previously reported to positively affect some properties of *Lactobacillus acidophilus* NCFM, such as bacteriocin production or growth rate, but not to significantly vary its adherence to mucin and HT29 cells [43]. In the present study, trehalose significantly decreased ($p < 0.05$) the ability of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 strain to adhere to both MUC and BSA. Only the microorganism adherence to COL was slightly increased by the addition of trehalose to the juice composition, and especially by further submitting the microbial strain to 100 MPa.

Regarding the application of high homogenization pressures, results obtained in this study also differ from those obtained by other authors [15,16] who confirmed that it can change the structure of microorganisms and increase their hydrophobicity, thus facilitating their adhesion to the cells of the digestive tract. Quite possibly, as documented by Patrignani et al. [44], response to HPH varies with the species and the characteristics of individual strains, as well as with the composition of the media containing the microorganism.

3.3. Storage of Fermented Clementine Juice

Changes in the microbial content of clementine juices fermented for 24 h with *Lactobacillus salivarius* spp. *salivarius* CECT4063 throughout 30 days of refrigerated storage at 4 °C were observed (Figure 4). HPH samples were not subjected to this study since the microorganism was not stressed by the application of a pressure gradient and so its tolerance to adverse conditions was expected to be similar to that of the microorganism that has grown in non HPH samples. In agreement with previous findings [15], the number of living cells remained constant during the first 3 days of storage and

began to decrease from the seventh day, probably due to nutrient and oxygen depletion and/or the excretion of organic acids and other biochemical contaminants into the medium. Zheng et al. [30] reported that *L. casei* was able to produce lactic acid even at refrigerated temperatures, thus reducing from 4.91 to 4.38 and from 5.31 to 4.48 the pH of fermented heat treated lychee juice and fermented HHP treated lychee juice, respectively, after storage for 28 days at 4 °C. In this study, no significant changes in the pH and Brix values of fermented clementine juice were observed after storage for 31 days at 4 °C ($p < 0.05$). It was not until day 21 when the counts reached values below the minimum concentration of 10^6 cfu/mL required to make an EU based health claim [27].

Statistical analysis on the loss of viability that occurred between the beginning and the end of storage revealed, with a confidence level of 95%, that the non HPH_0%T juice suffered the highest loss of viable cells (5.4 ± 0.5 $\log_{10}$ reduction on average). The simple addition of 10% (w/w) of trehalose to the juice formulation before its inoculation (non-HPH_10%T juice) slightly reduced the loss of viability to 4.92 ± 0.08 log10, regardless of the subsequent homogenization or not of the 24 h-fermented juice. On the contrary, the homogenization at 100 MPa significantly decreased the loss of viability of *Lactobacillus salivarius* CECT 4063 in the juices that did not include trehalose in their formulation (I+HPH_0%T) in the order of 4.4 ± 0.5 log10. Similar results were obtained by Betoret et al. [15] after storage of clementine juice rich in *Lactobacillus salivarius* spp. *salivarius* CECT 4063 for 10 days.

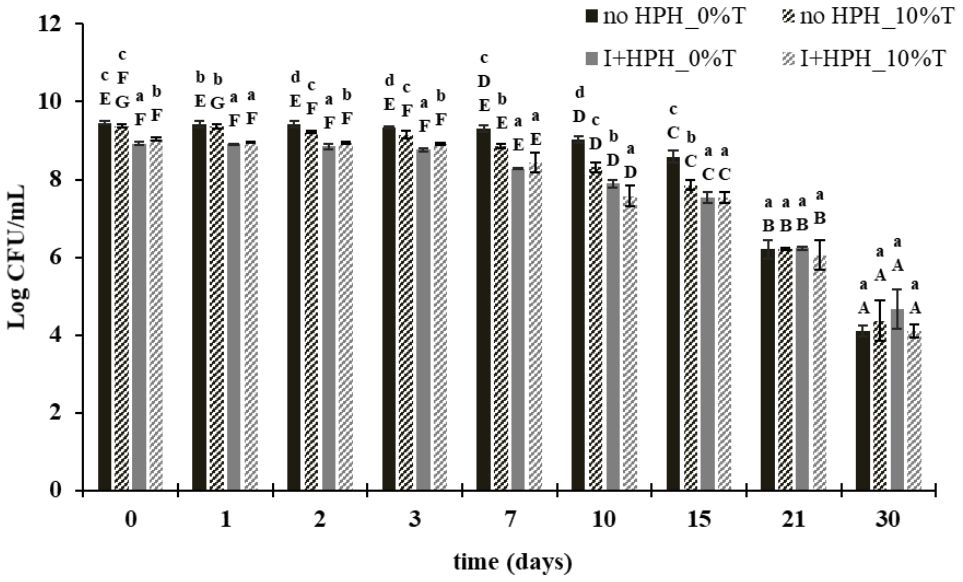

Figure 4. Microbial counts in clementine juice fermented with *L. salivarius* spp. *salivarius* CECT 4063 in cold storage for 30 days. Error bars represent the standard deviation of 4 replications. $^{a-d}$ at the same time and $^{A-G}$ in the same series indicate statistically significant difference with a 95% confidence level ($p < 0.05$).

Figure 5 shows the evolution in the antioxidant properties of 24 h fermented juices during storage in refrigeration for 30 days. Total phenolics and total flavonoids ranged

around a constant value, which turned out to be particular to each of the juices. A similar trend was observed for the ability to scavenge the radical ABTS+, which, excluding the no-table increase undergone within the first 24 h of storage and the drastic decline recorded on day 15, remained fairly stable. However, the antiradical activity measured by the DPPH method increased progressively throughout the storage period. This increase could be the result of the fermentative activity of the probiotic, which slowly continued under refrigeration conditions and allowed the formation of antioxidants compounds of a more hydrophobic nature and with a greater capacity to scavenge the radical DPPH•. In addition, the antioxidant capacity of phenolics could be enhanced by changes in their chemical structure taking place during the storage period [31].

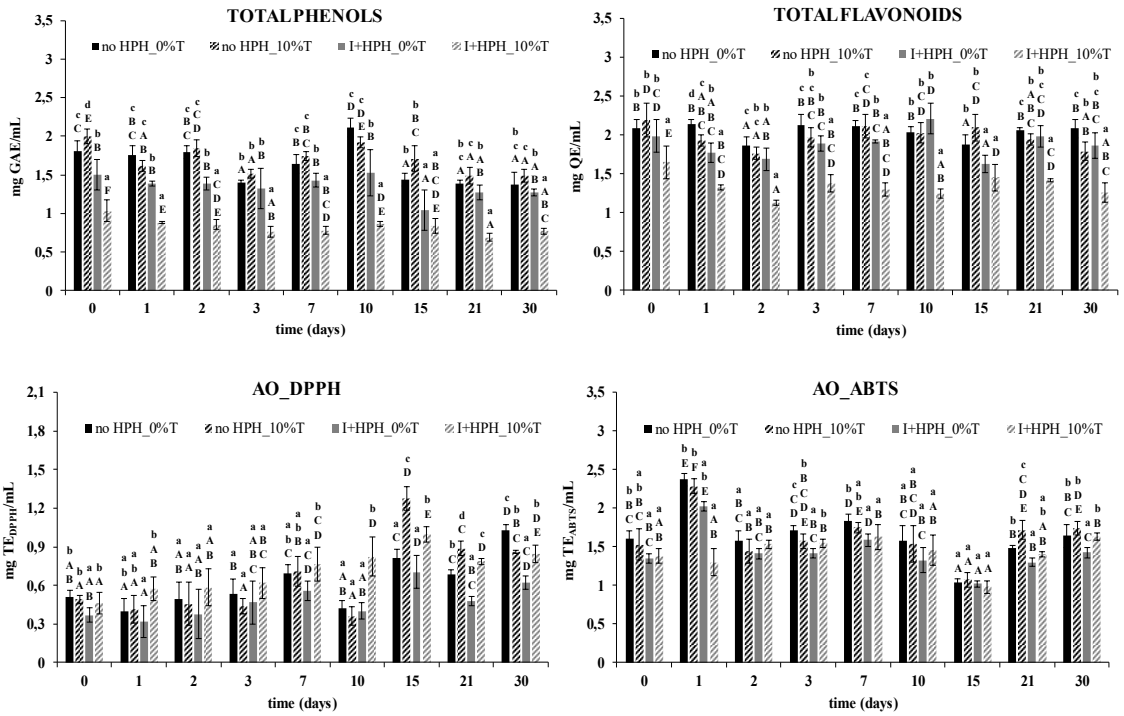

Figure 5. Antioxidant properties of clementine juice fermented with *L. salivarius* spp. *salivarius* CECT 4063 in cold storage for 30 days. Error bars represent the standard deviation of 4 replications. For each antioxidant property, $^{a-d}$ in the same time and $^{A-F}$ in the same series indicate statistically significant difference with a 95% confidence level ($p < 0.05$).

Setting the end of the shelf life based on the microbial counts, 24 h fermented juices should not be stored under refrigeration for more than 15 days in order to ensure the minimum concentration of 10^6 cfu/mL. Taking this into account, Table 3 shows the change in the antioxidant properties undergone by fermented clementine juices at the end of their shelf life. Results show that there was actually a significant decrease in all the antioxidant properties analysed. Only the antioxidant activity measured by the DPPH method improved remarkably at the end of the shelf life. Decline in total phenolic content was also observed in fermented heat and HHP treated lychee juice [31] and in fermented and unfermented sweet lemon juice [45] during 4 weeks of storage at 4 °C

and explained in terms of the oxidation degradation and the polymerization of phenolic compounds with proteins.

In general terms, degradation of both total phenols and total flavonoids was favoured by the homogenization at 100 MPa of the juice after 24 h of fermentation. This can be due to the reduction in particle size that results from homogenization, so that phenolic compounds, including flavonoids, were more sensitive to the factors responsible for their deterioration. In addition, these compounds were probably more available to be used as a substrate by the lactobacillus strain in order to continue with the fermentation. Decline in both total phenols and flavonoids in I+HPH samples was significantly reduced by the addition of 10% (w/w) of trehalose to the juice formulation before its inoculation. Such a protective effect of trehalose was also observed in non-HPH samples for the total phenolic content, while the decrease in total flavonoid content was enhanced in the presence of that disaccharide.

Table 3. Percentage of increase (values > 0) or decrease (values < 0) in the antioxidant properties undergone by 24 h-fermented clementine juices stored for 15 days at 4 °C.

Treatment		Total Phenols	Total Flavonoids	AO_DPPH	AO_ABTS
non-HPH	0%T	−23.1 ± 0.8 ^{b}	−7.1 ± 0.6 ^{c}	55 ± 11 ^{a}	−36 ± 3 ^{a}
	10%T	−15 ± 8 ^{c}	−8 ± 2 ^{c}	155 ± 19 ^{c}	−27 ± 4 ^{b}
I+HPH	0%T	−39 ± 2 ^{a}	−21.0 ± 1.2 ^{a}	107 ± 15 ^{b}	−24 ± 3 ^{b}
	10%T	−14 ± 2 ^{c}	−14.3 ± 0.7 ^{b}	117 ± 13 ^{b}	−27 ± 4 ^{b}

$^{a-c}$ in the same column indicate statistically significant differences with a 95% confidence level ($p < 0.05$). Mean value of 4 replications ± standard deviation.

Regarding the overall antioxidant capacity, the addition of trehalose, the homogenization and the combination of both factors resulted in a lower loss (as measured by the $ABTS^{+}$ radical method) or a greater increase (as measured by the $DPPH^{\bullet}$ radical method). The improvement in the ability to scavenge the free radical $DPPH^{\bullet}$ observed in the juices after 15 days of cold storage was significantly more evident in non-HPH samples than those include trehalose in their composition. Homogenization of the 24 h fermented juice reduced the protective effect attributed to trehalose on the total antioxidant capacity of the juices.

4. Conclusions

Fermentation of commercial clementine juice with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 for 24 h has a positive impact on antioxidant properties, while conferring a potential probiotic effect. Extending fermentation reduces the microbial population and does not significantly improve the antioxidant content. Homogenizing the juice before inoculation is not recommended, since despite it enhancing microbial growth, it favours antioxidant degradation. Adding 10% (*w*/*w*) of trehalose to juice formulation before inoculating and/or homogenizing the juice at 100 MPa after 24 h of fermentation has a negative impact on viable counts and does not significantly improve microbial strain ability to adhere to the proteins of the intestinal mucosa. However, both

techniques have been proven to be effective in preventing antioxidant oxidation and cell decay during storage of fermented juice under refrigeration, which should not last more than 15 days. Overall, it would not be necessary to add trehalose to the juice formulation or apply high homogenization pressures in order to get a 24 h-fermented liquid with maximum microbial and antioxidant contents throughout its shelf life.

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IV. CAPÍTULO 2

IV.2 Obtención de un snack de manzana (var. *Granny Smith*) con elevado contenido en compuestos antioxidantes y en unidades formadoras de colonias de *L. salivarius* CECT 4063

ARTÍCULO 3

Cristina Gabriela Burca-Busaga, Noelia Betoret, Lucía Seguí, Ester Betoret and Cristina Barrera (2020). Survival of *Lactobacillus salivarius* CECT 4063 and stability of antioxidant compounds in dried apple snacks as affected by the water activity, the addition of trehalose and high-pressure homogenization. Microorganisms, 8(8), 1095. DOI: 10.3390/microorganisms8081095. https://doi.org/10.3390/microorganisms8081095

ARTÍCULO 4

Cristina Gabriela Burca-Busaga, Noelia Betoret, Lucía Seguí, Jorge García-Hernández, Manuel Hernández and Cristina Barrera (2021). Antioxidants bioaccessibility and *Lactobacillus salivarius* (CECT 4063) survival following the *in vitro* digestion of vacuum impregnated apple slices: effect of the drying technique, the addition of trehalose, and high-pressure homogenization. Foods, 10(9), 2155. DOI: 10.3390/foods10092155. https://doi.org/10.3390/foods10092155

Como se ha comentado en apartados anteriores, los alimentos funcionales son aquellos que, además de nutrir, mejoran el estado de salud o incluso reducen el riesgo de padecer determinadas enfermedades. Entre los componentes responsables de estos beneficios se encuentran los probióticos, generalmente incorporados en matrices lácteas que no pueden ser consumidas por individuos con intolerancia a la lactosa o dislipemia, ni por aquellos que practican el veganismo. Como alternativa, surgen en el mercado otro tipo de bebidas probióticas a base de zumos de frutas y hortalizas, fermentados o no, que aportan otros compuestos bioactivos tales como fibra, vitaminas, aminoácidos, flavonoides y minerales. Además, mediante la técnica de impregnación a vacío, estas bebidas podrían introducirse en la matriz estructural de diversas frutas y hortalizas para dar lugar a productos enriquecidos que conservan su integridad celular y presentan unas propiedades similares a las del producto fresco, incluida su escasa durabilidad. Aunque la aplicación de una operación de deshidratación contribuiría en aumentar la estabilidad del producto impregnado, las condiciones de proceso deberían seleccionarse de manera que su impacto sobre la concentración de los compuestos de interés fuera mínimo. En el caso de los microorganismos con efecto probiótico, algunos estudios demuestran que el hecho de someterlos a situaciones de estrés moderado durante las fases iniciales de crecimiento aumenta su resistencia a las situaciones adversas a las que puedan someterse durante el procesado, el almacenamiento o el paso por el tracto gastrointestinal. Del tipo de secado también depende la estructura del producto final y, con ello, la protección que ésta confiera a los compuestos de interés y la facilidad con la que estos se liberen para poder ser absorbidos en el intestino delgado. Finalmente, la intensidad del secado condiciona la actividad del agua alcanzada al final del proceso que, para minimizar las reacciones de oxidación que degradan las proteínas y lípidos de las células microbianas, debería oscilar entre 0,1 y 0,3.

En este apartado se presentan los resultados obtenidos en los estudios conducentes a la obtención de un snack de manzana (var. *Granny Smith*) con elevado contenido en compuestos antioxidantes y en unidades formadoras de colonias de *L. salivarius* CECT 4063 mediante impregnación a vacío y posterior liofilización o secado con aire a 40 °C. En forma de dos artículos científicos, se analiza el efecto que la adición de un 10% (p/p) de trehalosa al zumo de clementina antes de su inoculación con el microorganismo y/o la homogeneización a 100 MPa del zumo de clementina fermentado ejercen sobre la viabilidad del lactobacilo y la estabilidad de los compuestos antioxidantes frente al procesado, el almacenamiento y la digestión simulada *in vitro*. A continuación, se detallan los resultados más relevantes:

- Tanto la liofilización como el secado con aire a 40 °C durante 12 y 24 h ejercieron un impacto negativo sobre el contenido en *Lactobacillus salivarius* CECT 4063, pero mejoraron las propiedades antioxidantes de las muestras impregnadas. De entre las técnicas de deshidratación empleadas, la liofilización resultó ser la que menos redujo la población microbiana, especialmente al añadir trehalosa al zumo de clementina previamente a su inoculación. Por otra parte, el secado hasta una actividad del agua de 0,45 con aire a 40 °C de las muestras impregnadas con el líquido que no incluía trehalosa en su composición y/o no se

homogeneizó a 100 MPa produjo un mayor incremento en el contenido en compuestos fenólicos (incluidos los del tipo flavonoides) y en la capacidad para secuestrar el radical DPPH•.

- La población microbiana en los snacks de manzana disminuyó tras 30 días de almacenamiento a temperatura ambiente. De entre las muestras analizadas, las obtenidas mediante secado con aire a 40 °C hasta una actividad del agua de 0,35 fueron las que menor reducción experimentaron en la población microbiana, especialmente tras añadir trehalosa al líquido de impregnación, seguidas muy de cerca por las muestras liofilizadas hasta una actividad del agua de 0,25. De ello se deduce que la estabilidad microbiana no depende exclusivamente de la actividad del agua alcanzada en el producto final, sino también de su estructura. En este caso, además, el secado con aire a 40 °C durante 12 h pudo inducir la formación de proteínas como reacción al estrés térmico.
- Con respecto al contenido en compuestos con actividad antioxidante, solo la pérdida de fenoles totales tras un mes de almacenamiento aumentó significativamente con la actividad del agua de los snacks de manzana, mientras que la pérdida de fenoles totales y la capacidad para secuestrar el radical DPPH• se vieron más afectados por la composición del líquido de impregnación, la técnica de deshidratación empleada y la interacción entre ambos factores. En cualquier caso, los compuestos antioxidantes resultaron ser más estables frente al almacenamiento en el caso de las muestras de manzana liofilizadas.
- De los estudios de digestión *in vitro* se deduce que tanto la supervivencia de *Lactobacillus salivarius* CECT 4063 como la bioaccesibilidad de los antioxidantes se vieron afectadas por la matriz alimentaria de la que forman parte.
- La viabilidad del microorganismo tras la digestión *in vitro* resultó máxima al incorporarlo en la matriz sólida de láminas de manzana mediante la técnica de impregnación a vacío, pero disminuyó significativamente con la posterior deshidratación.
- La bioaccesibilidad de los compuestos antioxidantes también alcanzó los valores más elevados durante la digestión *in vitro* de las muestras impregnadas a vacío.
- La adición de trehalosa al zumo de clementina antes de su inoculación y la homogeneización a 100 MPa del zumo fermentado aumentaron notablemente la bioaccesibilidad de los antioxidantes totales en las muestras liofilizadas y en las secadas con aire a 40 °C y la bioaccesibilidad de los flavonoides totales en las muestras impregnadas a vacío.
- La homogeneización a 100 MPa del zumo de clementina fermentado empleado como líquido de impregnación también aumentó la supervivencia del *Lactobacillus salivarius* CECT 4063 durante la digestión *in vitro* de las muestras de manzana liofilizadas.
- Aunque los compuestos con actividad antioxidante alcanzaron una mayor concentración en las muestras deshidratadas que en las simplemente impregnadas, los cambios estructurales asociados a las operaciones de secado y liofilización disminuyeron su bioaccesibilidad de manera que la concentración

que alcanzaron al final del proceso de digestión *in vitro* resultó muy similar a la obtenida tras la digestión de las muestras impregnadas.

En conclusión, se puede afirmar que la impregnación a vacío con zumo de clementina fermentado con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 es una técnica adecuada para incorporar microorganismos con potencial efecto probiótico a la matriz estructural de láminas de manzana en cantidad suficiente como para poder ejercer un efecto beneficioso sobre la salud del consumidor. De entre las diferentes técnicas de deshidratación aplicadas para aumentar la estabilidad del producto impregnado, la liofilización sería la más recomendada pues permite mantener los recuentos por encima de 10^7 UFC/g al final del almacenamiento y tras la digestión *in vitro*. Como una alternativa de procesado más económica también se podría aplicar un secado con aire a 40°C, lo que resultaría en un producto con unas propiedades organolépticas totalmente diferentes. En tal caso, lo recomendable sería añadir un 10% en peso de trehalosa al zumo de clementina antes de su inoculación y prolongar el secado hasta valores de actividad del agua en torno a 0,35 para lograr una concentración microbiana aceptable y que se mantenga estable durante el almacenamiento del producto deshidratado, aunque difícilmente esta población se liberaría a los fluidos gástricos e intestinales durante la digestión. Aunque la actividad antioxidante de las láminas de manzana impregnadas mejora significativamente tras la liofilización o el secado con aire a 40°C, la baja estabilidad mostrada por los compuestos responsables de la misma durante el almacenamiento, unida a su escasa bioaccesibilidad, confirman que la ingesta del producto impregnado sea, a pesar de su elevada perecibilidad, la opción más saludable.

ARTÍCULO 3

SURVIVAL OF *LACTOBACILLUS SALIVARIUS* CECT 4063 AND STABILITY OF ANTIOXIDANT COMPOUNDS IN DRIED APPLE SNACKS AS AFFECTED BY THE WATER ACTIVITY, THE ADDITION OF TREHALOSE AND HIGH-PRESSURE HOMOGENIZATION

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Abstract: Survival of probiotic microorganisms in dried foods is optimal for water activity (a_w) values between 0.1 and 0.3. Encapsulating and adding low-molecular weight additives can enhance probiotic viability in intermediate a_w food products, but the effectiveness of sub-lethal homogenization is still not proven. This study evaluates the effect of 10% (w/w) trehalose addition and/or 100 MPa homogenization on *Lactobacillus salivarius* CECT 4063 counts and antioxidant properties of apple slices dried to different water activity values (freeze-drying to a a_w of 0.25 and air-drying at 40 °C to a a_w of 0.35 and 0.45) during four-week storage. Optical and mechanical properties of dried samples were also analysed. Freeze-drying had the least effect on the microbial counts and air drying at 40 °C to a a_w of 0.35 had the greatest effect. Antioxidant properties improved with drying, especially with convective drying. Decreases in both microbial and antioxidant content during storage were favoured in samples with higher water activity values. Adding trehalose improved cell survival during storage in samples with a water activity of 0.35, but 100 MPa homogenization increased the loss of viability in all cases. Air-dried samples became more translucent and reddish, rather rubbery, and less crispy than freeze-dried ones.

Keywords: *Lactobacillus salivarius* spp. *salivarius*; water activity; trehalose; high pressure homogenization; antioxidants; hot air drying; freeze-drying

1. Introduction

Functional foods are those that, beyond their nutritional value, benefit human health by improving it, and even reducing the incidence of certain diseases. This includes everything from fruits and vegetables, with a naturally high content of antioxidant compounds [1], to design foods, in which one or more ingredients have been added, removed, concentrated, or diluted.

Among fruits, apples are one of the richest in active compounds, mainly pectin (which acts as soluble fiber), amino acids, flavonoids (catechins and quercetin) and minerals (calcium, iron, magnesium, phosphorus, and potassium), which provide apples with anti-inflammatory, antidiarrheal and anticancer properties, among others [2]. This, coupled

with the high porosity of apples [3], has led to several studies in recent years aimed at obtaining apple snacks fortified with calcium [4], iron [5], antioxidants [6] and even probiotic microorganisms [7-9].

Probiotics are defined as ”live microorganisms which when administered in adequate amounts (10^8–10^9 CFU/day) confer a health benefit on the host” [10]. The major bacterial strains showing probiotic properties are lactic acid bacteria (LAB) from *Lactobacillus* and *Bifidobacterium* spp., which have been accorded the Generally Recognized as Safe (GRAS) status. The various health benefits documented for probiotics include suppression of obesity and type 2 diabetes through modulating glucose metabolism and prevention of liver disease, as well as suppression of hypercholesterolemia and cardiovascular disease through modulating total cholesterol, LDL-cholesterol, and triglyceride metabolisms [11]. Other health disorders, such as allergic inflammation and atopic dermatitis; colon, bladder, and cervical cancers; inflammatory bowel disease; diarrhea; lactose intolerance; renal diseases; hormonal immune response failure and poisoning caused by toxic compounds were also reported to improve with the ingestion of specific probiotic strains. Since viability of probiotic bacteria is required in order to exert health benefits in humans, ensuring that the different probiotic strains used in the formulation of foods are resistant to food manufacturing and storage conditions, as well as to the gastrointestinal digestion of the food, is a basic requirement when developing probiotic foods. Along with the processing temperature and the oxygen content in the product, the water activity (aw) is a key factor in maintaining probiotic viability in dry products [12–17]. As reported by several authors [12,13,15,16], viability of probiotic strains is lost rapidly with aw > 0.25. They indicate optimal water activity values to be between 0.1 and 0.3, at which water might interact with functional groups and block reaction sites, thus avoiding interaction with oxygen and oxidation reactions that cause degradation of lipids and proteins of the probiotic cell. However, reaching such low water activity values is not an easy task in the case of fruits, due to their relatively high soluble solids content. In such cases, encapsulation [16,18] or the addition of various low-molecular weight additives (inulin, Arabic gum, trehalose, sucrose, maltodextrin, etc.) [12,14,17,19-21] have been reported to significantly enhance the probiotic viability in intermediate water activity food products. Other techniques, such as the application of sub-lethal homogenization pressures (20–100 MPa) were also observed to increase the survival of probiotic strains in adverse conditions by inducing changes in the hydrophobicity of the cell membrane or in the availability of nutrients [22,23].

According to everything discussed above, this study aims to evaluate the effect that the drying technique and the water activity reached by the dried product has on the survival of Lactobacillus salivarius spp. salivarius and the stability of antioxidant properties of apple-based probiotic snacks during four-week storage under controlled conditions.

2. Materials and Methods

2.1. Raw Materials

Apples (*Malus domestica* cv. *Granny Smith*) used in the present study were purchased from a local market in Valencia, Spain. This variety was chosen for its high homogeneity and porosity, compared to other fruits. After being washed with tap water, apples were cut, following the longitudinal axis, into 5-mm-thick rings of 20 mm and 65 mm internal and external diameter, respectively.

Lactobacillus salivarius spp. *salivarius* supplied by the Spanish Culture Type Collection (strain CECT 4063, University of Valencia, Valencia, Spain) was selected as a probiotic microorganism in this study due to its potential effect against the infection caused by Helicobacter pylori and its ability to adapt to the apple solid matrix [8]. Revival of the lyophilized strain was carried out in MRS Broth at 37°C, following supplier's recommendations. Then, the stock culture was plated on MRS Agar and incubated at 37 °C for 24 h. During the experiments, the plates were stored at 4 °C. When required, the white layer grown on the surface of each plate was collected in a test tube containing 9 mL of sterile MRS Broth and was further incubated at 37°C for 24 h in order to obtain inoculums with a *Lactobacillus salivarius* spp. *salivarius* concentration on the order of $(7.7 \pm 0.9) \cdot 10^8$ CFU/mL.

Commercial clementine juice (Hacendado brand, Valencia, Spain) was used in the impregnation liquid preparation. Following the procedure of Betoret et al. [8], 5 g/L of yeast extract and 9.8 g/L of sodium bicarbonate (both supplied by Scharlab, S.L., Barcelona, Spain) were added to the juice in order to ensure the appropriate growth of the microbial strain. One hundred milligrams per gram of food-grade trehalose from tapioca starch (TREHA TM, Cargill, SLU, Barcelona) were added in half of the preparations. After stirring at 200 rpm to get all the ingredients dissolved, the liquids were inoculated with 4 mL/L of MRS Broth containing the microorganism and incubated at 37°C for 24 h to grow the acid lactic bacteria. Then, half of the liquid was homogenized at 100 MPa in a laboratory-scale high pressure homogenizer (Panda Plus 2000, GEA-Niro Soavi, Parma, Italy) and the other half was used directly in the vacuum impregnation step. Four different impregnation liquids were prepared in total: 0%_0MPa, 0%_100MPa, 10%_0MPa and 10%_100MPa, the percentage referring to the addition of trehalose and MPa to the homogenization pressure applied.

2.2. Snack Manufacturing Process and Storage

Vacuum impregnation (VI) was first carried out in a vacuum chamber (Heraeus Vacuum Oven, Thermo Fisher Scientific Inc.) connected to a vacuum pump (Ilmvac, Gardner Denver Thomas GmbH Welch Vacuum, Fürstenfeldbruck, Germany.) by applying a vacuum pressure of 50 mbar for 10 min to the apple rings immersed in the corresponding impregnation liquid (1:4 mass ratio) and then restoring the atmospheric pressure for another 10 min. Once impregnated, apple samples were stabilized by freeze-drying (FD) or convective drying at 40 °C until a water activity value of 0.45 or 0.35 (AD_0.45 and AD_0.35, respectively) was reached.

Freeze-drying was performed in two steps: freezing at −40 °C for 24 h in a CVN-40/105 Matek freezer and further sublimation at −45 °C and 0.1 mbar for 24 h in a 6–80 Telstar Lioalfa pilot plant scale freeze-dryer.

Convective drying was carried out in a CLW 750 TOP+ tray dryer (Pol-Eko-Aparatura SPJ, Vladislavia, Poland) with a cross flow of air at 2 m/s and 40 °C for about 24 or 48 h, depending on the water activity required at the end of the process.

Both freeze-dried and air-dried apple snacks were stored at room temperature in opaque and airtight bags for 4 weeks.

2.3. Analytical Determinations

2.3.1. Moisture Content and Water Activity

Moisture content of apple samples was measured following the AOAC official method 934.06-1934 for dried fruits [24] and water activity was measured at 25 °C in a previously calibrated dew point hygrometer (Decagon Aqualab model CX-2, Pullman, Washington, USA), with an accuracy of ± 0.003).

2.3.2. Antioxidant Properties

Antioxidant properties were determined for the extracts obtained by mixing a certain amount of apple (2 g vacuum impregnated apple and 0.35 g for freeze-dried and air-dried apple) with 10 mL of an 80:20 (v/v) methanol of analytical high-performance liquid chromatography grade (Merck KGaA and affiliates, Darmstadt, Germany) in water solution. After dispersing with a T 25 digital ULTRA-TURRAX®, the mixture was stirred in the dark at 200 rpm for 1 h and centrifuged at 10,000 rpm for 5 min at 4 °C in a Thermo Fisher Scientific Megafuge 16 centrifuge so as to obtain the methanolic extracts used in the antioxidant compounds quantification.

Total phenol content was determined following the method described by Singleton and Rossi [25] with some modifications. Briefly, 125 µL of the extract, 500 µL of bidistilled water and 125 µL of Folin–Ciocalteu phenol reagent (Merck KGaA and affiliates, Darmstadt, Germany) were mixed in a spectrophotometric cell. After incubation for 6 min in the dark, 1.25 mL of 7% Na2CO3 and 1 mL of bidistilled water were added. The mixture was kept at room temperature for 90 min in the dark and then the absorbance was measured at 760 nm on a Helios Zeta UV/Vis Thermo Scientific spectrophotometer (Waltham, Massachusetts, USA). Results were expressed as mg of gallic acid equivalents (purity ≥ 98%, Merck KGaA and affiliates, Darmstadt, Germany) per gram of dried sample by comparison with a standard calibration curve prepared in the range between 100 and 500 ppm ($y = 0.0032x + 0.0305$; $R2 = 0.9963$)

Total flavonoid content was determined according to the method described by Luximon-Rama et al. [26] with some modification. For this, 1.5 mL of the extract and 1.5 mL of a 2% (w/v) aluminium chloride (purity ≥ 98%) in methanol of analytical high-performance liquid chromatography-grade solution (both supplied by Merck KGaA and affiliates, Darmstadt, Germany) were mixed in a spectrophotometric cuvette. After 10 min

reacting in the dark, the absorbance at 368 nm was measured on a Helios Zeta UV/Vis Thermo Scientific spectrophotometer. Results were expressed as mg of quercetin equivalents (purity ≥ 95%, Merck KGaA and affiliates, Darmstadt, Germany) per gram of dried sample by comparison with a standard calibration curve prepared in the range between 12.5 and 200 ppm (y = 0.0095x + 0.0799; R2 = 0.9977).

Finally, antioxidant activity of apple extracts was quantified according to their ability to scavenge the DPPH$^{\bullet}$ radical, as reported by Brand-Williams et al. [27]. Basically, 50 µL of the extract and 2950 µL of a 0.06 mM DPPH-methanol solution were mixed in a spectrophotometric cuvette and kept for 90 min in the dark before measuring the absorbance at 515 nm on a Helios Zeta UV/Vis Thermo Scientific spectrophotometer. Results were expressed as mg of Trolox equivalents (purity ≥ 97%, Merck KGaA and affiliates, Darmstadt, Germany) per gram of dried sample by comparison with a standard calibration curve prepared in the range between 100 and 300 ppm (y = −0.0014x + 0.4949; R2 = 0.9981).

2.3.3. Colour Measurements

Optical properties of apple samples were measured on a black background with a spectrocolorimeter (Minolta, CM-3600d), using D65 as illuminator and as 10 ° observer. Colour measurements were given in CIE L*a*b* coordinates, where L* is the lightness that varies from black (0) to white (100), a* is the green (−) to red (+) colour component and b* is the blue (−) to yellow (+) colour component.

2.3.4. Mechanical Properties

Mechanical properties of apple samples were evaluated by means of a puncture test in a TA-XT Plus texturometer (Stable Micro Systems, Godalming, United Kingdom). The test was carried out with a 2 mm diameter stainless steel cylindrical probe (Stable Micro Systems, Godalming, United Kingdom) that passed completely through the sample at a rate of 2 mm/s. From the force vs. distance curves provided by the equipment, the maximum force (F_{max}, in N) and the distance travelled by the punch to reach the maximum force (d_{max}, in mm) were obtained.

2.3.5. Microbial Counts

Lactobacillus salivarius spp. *salivarius* CECT 4063 content in both liquid and solid samples were estimated by serial dilution from 10−1 to 10−6 with peptone water, inoculation onto MRS Agar and incubation at 37 °C for 24 h. In the case of apple samples, the first dilution was obtained in a stomacher bag by mixing 5 g of sample with 45 mL of sterile peptone water (dilution 10−1) and blending at medium speed for 2 min.

2.3.6. Statistical Analysis

Statistical analysis was carried out with the Statgraphics Centurion XVI program (Stable Micro Systems, Godalming, United Kingdom) by means of simple and multivariate analysis of variance (ANOVA) with a 95% confidence level.

3. Results and Discussion

3.1. Survival of *Lactobacillus salivarius* spp. salivarius during the Snack Manufacturing Process

Table 1 shows the *Lactobacillus salivarius* spp. *salivarius* content of the different impregnation liquids, apple slices impregnated with each of them and subsequently freeze-dried or air-dried until reaching final water activity value. To better indicate the potential probiotic character of the different samples analysed, concentrations were expressed on a wet basis instead of a dried basis. Together with experimentally obtained counts, theoretically calculated ones from the application of a mass balance in steady state, neglecting the generation term, were also calculated (equations 1 to 3).

$$[x^{mic}_{VI\,APP}]_{THEO} = \frac{X \cdot (1/\rho_{APP}) \cdot [x^{mic}_{VI\,LIQ}]_{EXP}}{1 + X \cdot (\rho_{VI\,LIQ}/\rho_{APP})} \quad (1)$$

$$[x^{mic}_{DEH\,APP}]_{THEO} = \frac{m_{VI\,APP} \cdot [x^{mic}_{VI\,APP}]_{EXP}}{m_{DEH\,APP}} \quad (2)$$

where $[x^{mic}_{VI\,APP}]_{THEO}$ and $[x^{mic}_{DEH\,APP}]_{THEO}$ are the theoretically calculated microbial contents of vacuum impregnated and dehydrated apples, respectively (CFU/g); $[x^{mic}_{VI\,LIQ}]_{EXP}$ and $[x^{mic}_{VI\,APP}]_{EXP}$ are the experimentally obtained microbial contents of impregnation liquids (CFU/mL) and vacuum impregnated samples (CFU/g), respectively; X is the impregnated volume fraction (reported by Fito et al. [3] to be 19 ± 1.5 m^3 impregnation liquid/m^3 fresh sample); $\rho_{VI\,LIQ}$ is the density of the impregnation liquid (reported by Betoret et al. [8] to be 1.085 ± 0.002 g/cm^3); ρ_{APP} is the apparent density of fresh apple (reported by Fito et al. [3] to be 0.802 ± 0.010 g/cm^3); $m_{VI\,APP}$ and $m_{DEH\,APP}$ are the total mass of vacuum impregnated and dehydrated apples, respectively (g); and $x^{w}_{VI\,APP}$ and $x^{w}_{DEH\,APP}$ are the experimentally measured moisture contents of vacuum-impregnated and dehydrated apples, respectively (g w/g).

Comparison between experimental and predicted values gives information about the influence that the different stages exert on the microbial counts, depending on the type of impregnation liquid used, which is expressed as a log reduction in Table 1. Starting with liquid samples, a significant increase in the microbial content was observed by adding 10% (w/w) of trehalose to the juice before inoculation (10%_0MPa) or by homogenizing at 100 MPa the fermented juice (0%_100MPa). From this it follows that, as previously stated by other authors [28,29], trehalose serves as an important carbon and energy source, and as long as the osmotic pressure created in the growing media was not too high, it has the ability to enhance the growth status of several lactic acid bacteria strains. As regards the juice homogenization at sub-lethal pressures, it was previously reported to increase the microbial counts by reducing the size of the cloud particles and thus favouring the availability of nutrients [23]. In contrast, the combination of these two factors (10%_100MPa) did not significantly improve the viable counts.

Table 1. Microbial counts in vacuum impregnating liquids (VI LIQ) and apple samples: comparison between predicted and experimental values. VI APP stands for vacuum impregnated apples, FD stands for freeze-dried apples, AD_0.45 stands for apples air-dried until reaching a water activity value aw ≈ 0.45 and AD_0.35 stands for apples air-dried until reaching a aw ≈ 0.35. Mean value of three replicates ± standard deviation. Different letters in the same column indicate statistically significant differences with a 95% confidence level (p-value < 0.05).

SAMPLE	TRE%_HPH	*Log CFU/g*	
		Experimental	***Log Reduction***
VI LIQ	0%_0MPa	8.52 ± 0.02^{fgh}	-
	10%_0MPa	9.1 ± 0.2^{i}	
	0%_100MPa	8.94 ± 0.12^{hi}	
	10%_100MPa	8.48 ± 0.07^{fg}	
VI APP x^w = 85.3 ± 1.2 g w/100 g	0%_0MPa	7.839 ± 0.009^{de}	0.10 ± 0.02^{j}
	10%_0MPa	8.20 ± 0.11^{ef}	-0.09 ± 0.04^{i}
	0%_100MPa	8.1 ± 0.2^{ef}	-0.08 ± 0.04^{i}
	10%_100MPa	7.7 ± 0.5^{cd}	-0.05 ± 0.02^{i}
FD x^w = 5.0 ± 1.0 g w/100 g	0%_0MPa	8.12 ± 0.10^{ef}	-0.53 ± 0.02^{g}
	10%_0MPa	8.74 ± 0.06^{ghi}	-0.22 ± 0.04^{h}
	0%_100MPa	8.39 ± 0.04^{fg}	-0.64 ± 0.08^{g}
	10%_100MPa	7.68 ± 0.08^{cd}	-0.9 ± 0.3^{f}
AD_0.45 x^w = 11.4 ± 1.5 g w/100 g	0%_0MPa	7.46 ± 0.05^{c}	-1.167 ± 0.009^{e}
	10%_0MPa	8.26 ± 0.03^{ef}	-1.46 ± 0.03^{cd}
	0%_100MPa	8.217 ± 0.004^{ef}	-1.60 ± 0.07^{c}
	10%_100MPa	7.80 ± 0.04^{cde}	-1.25 ± 0.02^{de}
AD_0.35 x^w = 9.2 ± 0.8 g w/100 g	0%_0MPa	7.5 ± 0.3^{cd}	-1.337 ± 0.009^{de}
	10%_0MPa	6.25 ± 0.08^{a}	-2.68 ± 0.03^{a}
	0%_100MPa	7.02 ± 0.03^{b}	-2.10 ± 0.07^{b}
	10%_100MPa	6.10 ± 0.02^{a}	-2.63 ± 0.02^{a}

As regards the *Lactobacillus salivarius* spp. *salivarius* content of vacuum impregnated samples, a reduction of around 0.8-log10 compared to that of vacuum impregnation liquids was observed. This result was expected because the external liquid is known to fill around 20% of the initial volume of apple slices during the vacuum impregnation step [3]. Furthermore, the similarity between predicted and experimentally obtained values for vacuum-impregnated apples (log reduction values close to zero) proves that vacuum impregnation is a useful technique for incorporating probiotics into a food solid matrix without negatively affecting their viability [30,8,9]. In accordance with the microbial counts reported for the impregnation liquids, apples impregnated with liquids 10%_0MPa and 0%_100MPa reached the highest content in *Lactobacillus salivarius* spp. *salivarius* after the vacuum impregnation step.

Finally, because of the water loss taking place during the dehydration step, the microbial content of dried apple samples was generally higher than that of the corresponding impregnated ones. However, based on the differences between predicted and experimental values, this increase was not as high as expected, thus showing the negative impact that drying has on the viability of the *Lactobacillus salivarius* spp. *salivarius* CECT 4063 strain. Among the drying techniques applied, freeze-drying had the least influence on the *Lactobacillus salivarius* spp. *salivarius* survival (0.6 ± 0.3-log10 reduction on average), and air drying at 40 °C until reaching a water activity around 0.35

had the greatest influence (2.2 ± 0.6-log10 reduction on average). This is mainly due to the lower exposure to oxygen and high temperature in the case of comparing freeze-drying with air drying, and to the shorter drying duration in the case of comparing air drying until reaching a water activity around 0.35 (48 h) with air drying until reaching a water activity around 0.45 (24 h). The loss of cell viability observed after freeze-drying might mainly be due to intracellular ice formation, which can damage the probiotic cell membrane [31]. The number of cells that survived the freeze-drying step ranged between 14.2% ± 0.7% and 61% ± 6% in apple samples impregnated with liquids 10%_100MPa and 10%_0MPa, respectively.

In a similar study carried out with *Lactobacillus salivarius* spp. *salivarius* (UCC 500) solutions containing different substances as protective media [32], the survival rate immediately after freeze-drying was reported to increase from 4% in water without any protective agent to 34% in a suspension containing 4% of trehalose or even up to 83%–85% in a suspension containing 4% of sucrose, 4% of trehalose and 18% of skimmed milk. These results also suggest that, as previously indicated by Betoret et al. [33], the inclusion of only the probiotic into the apple's porous structure by means of vacuum impregnation might confer it with protection against cell damage caused by freezing and subsequent sublimation of frozen water.

This statement is not only supported by the higher viability obtained in the present study when the microorganism was subjected to freeze-drying as part of the apple porous structure (47% in apples impregnated with liquid 0%_0MPa) compared to that reported by Zayed and Roos [32] for the freeze-drying of the microorganism in just water, but also by the fact that a lower concentration of trehalose (≈ 2% w/w in the liquid phase of apples impregnated with liquid 10%_0MPa) resulted in a considerably higher survival of the microorganism to the freeze-drying step (78%) compared to that reported by Zayed and Roos [32] for the freeze-drying of the microorganism in a suspension containing 4% of trehalose.

It is also possible that, since trehalose cannot be naturally synthetized by lactic acid bacteria [34], the beneficial effect of trehalose during freeze-drying was enhanced by its addition to the growth medium. Under less favourable drying conditions, as in the case of air-drying, the addition of 10% (w/w) of trehalose to the clementine juice before microbial inoculation resulted in less or no efficiency at all in preventing cell death, depending on the length of time exposure.

Regarding the homogenization of the fermented juice at 100 MPa, its effect on the CECT 4063 strain's survival to drying was only significant when drying apple samples with air until reaching a water activity around 0.45 (AD_0.45 samples). This might be due to particle size reduction and related increased bioavailability of main compounds with antioxidant activity present in the juice [35]. As a result, probiotic cells would be more protected against oxidative stress caused by contact with the oxygen from the drying air. Likewise, this protective effect would not be as evident either in those samples not exposed to oxidation during drying, or in those samples exposed to excessive oxygen during drying. In such cases, the stress caused by the pressure gradient applied to the

system seemed to be more harmful than beneficial for the probiotic survival to the drying process.

Besides the previous discussion, *Lactobacillus salivarius* spp. *salivarius* counts in dried apples were in all cases higher than 10^6 CFU/g, which is the minimum concentration generally accepted for the probiotic benefits to be transferred to the consumer [36].

3.2. Antioxidant Properties Affected by the Snack Manufacturing Process

Antioxidant properties of apple slices subjected to vacuum impregnation with the different impregnation liquids and subsequently freeze-dried or dried with air at 40 °C until reaching a final water activity value are shown in Table 2.

Table 2. Antioxidant properties of apple samples after each stage of the snack manufacturing process. VI APP stands for vacuum impregnated apples, FD stands for freeze-dried apples, AD_0.45 stands for apples air-dried until reaching a aw ≈ 0.45 and AD_0.35 stands for apples air-dried until reaching a aw ≈ 0.35. Mean value of three replicates ± standard deviation. Different letters in the same column indicate statistically significant differences with a 95% confidence level (p-value < 0.05).

SAMPLE	TRE%_HPH	*Total Phenols (mg GAE/g dw)*	*Total Flavonoids (mg QE/g dw)*	*Antioxidant Activity (mg TE/g dw)*
VI APP a_w = 0.983 ± 0.002	0%_0MPa	5.4 ± 0.7^{a}	1.24 ± 0.15^{abc}	7.0 ± 0.9^{bcdef}
	10%_0MPa	5.7 ± 1.2^{abc}	1.07 ± 0.06^{a}	7 ± 3^{bcde}
	0%_100MPa	5.7 ± 0.5^{ab}	1.3 ± 0.3^{abc}	6.0 ± 0.9^{bc}
	10%_100MPa	5.3 ± 1.5^{a}	1.2 ± 0.4^{ab}	6.4 ± 1.8^{bcd}
FD a_w = 0.25 ± 0.02	0%_0MPa	5.4 ± 0.3^{ab}	1.584 ± 0.013^{cd}	8.64 ± 0.07^{efghi}
	10%_0MPa	8.39 ± 0.03^{f}	1.187 ± 0.013^{abc}	10.3 ± 0.4^{i}
	0%_100MPa	6.3 ± 0.3^{abcd}	1.76 ± 0.12^{de}	4.9 ± 0.3^{ab}
	10%_100MPa	6.6 ± 0.5^{abcde}	1.74 ± 0.03^{de}	3.6 ± 0.5^{a}
AD_0.45 a_w = 0.42 ± 0.02	0%_0MPa	10.6 ± 0.3^{g}	2.47 ± 0.09^{g}	10.2 ± 0.4^{i}
	10%_0MPa	6.7 ± 0.2^{bcde}	1.51 ± 0.06^{bcd}	9 ± 2^{ghi}
	0%_100MPa	7.6 ± 0.2^{def}	2.13 ± 0.09^{efg}	6.9 ± 0.2^{bcdefg}
	10%_100MPa	7.1 ± 0.5^{cdef}	1.53 ± 0.02^{cd}	8.5 ± 0.4^{defghi}
AD_0.35 a_w = 0.36 ± 0.03	0%_0MPa	$8.1 \pm 0.\ 2^{ef}$	1.990 ± 0.006^{ef}	9.2 ± 0.3^{fghi}
	10%_0MPa	6.6 ± 0.5^{abcde}	1.876 ± 0.012^{de}	7.68 ± 0.15^{cdefgh}
	0%_100MPa	7.8 ± 0.4^{def}	2.36 ± 0.07^{fg}	6.20 ± 0.07^{bcde}
	10%_100MPa	5.867 ± 0.014^{abc}	1.044 ± 0.005^{a}	9.5 ± 0.3^{hi}

As can be seen, the different liquids used in the vacuum impregnation step did not significantly affect either the total phenol and flavonoid content, or the overall antioxidant activity measured by the DPPH assay of vacuum-impregnated samples (VI APP). Antioxidant properties of apple slices generally improved after the dehydration step, but to a different extent, depending on the technique and the impregnation liquid previously used. Although slightly less pronounced, the increase in total phenol and flavonoid content and in the DPPH scavenging ability observed in freeze-dried samples could be explained in terms of a more efficient extraction of the antioxidant compounds. In fact, freeze-drying is often included in analytical procedures for use prior to the extraction of bioactive compounds from fruit matrices, while reducing the risk of isomerization and other undesirable reactions caused by their high enzymatic content

[37]. Homogenization significantly increased total flavonoid release from the freeze-dried apple structure, but hardly affected that of total phenols, and even hindered that of other antioxidant compounds. It follows from this that particle size reduction and the release of antioxidant compounds from the complex structures in which they are retained due to high pressure homogenization [35] was particularly high in the case of total flavonoids. Meanwhile, the addition of trehalose to the juice formulation significantly increased total phenol and antioxidant release from the freeze-dried apple structure but hindered that of total flavonoids. To explain this, reference can be made to the ability of trehalose to capture free radicals and prevent them from reacting with the reagents used in their analytical determination [22].

In the case of samples dried with air at 40°C, moderate heating might have promoted the generation of new bioactive compounds or compounds with a greater antioxidant activity, as was reported for lycopene and β-carotene in cherry tomato halves undergoing osmotic dehydration at both 30°C and 40°C [38] or to polyphenols from granulated jaggery solutions subjected to heating below 100 °C for less than 20 min [39]. Although oxidation by long contact with the air stream was expected to cause antioxidant degradation, it might be negligible in this case compared to the aforementioned synthesis. Regarding the impregnation liquid used, neither the addition of trehalose to its composition nor the homogenization significantly improved the antiradical properties of air-dried apple snacks. However, the slight decrease observed in total phenol and flavonoid content when extending the drying time to reach a lower water activity was negligible (for total phenol content) or turned into a notable increase (for total flavonoid content) in those apples impregnated with liquids 0%_100MPa and 10%_0MPa. Impregnation of apple slices with liquids 0%_100MPa and 10%_0MPa also prevented Maillard reactions, which were reported to promote the formation of novel compounds having DPPH radical-scavenging activity [40]. On the contrary, combining the two factors in the same impregnation liquid seemed to favour both polyphenol degradation and Maillard reactions, as deduced from the significant lower values in total phenol and flavonoid content and the higher antioxidant activity obtained for AD_0.35 samples impregnated with the liquid 10%_100MPa.

3.3. Colour Properties as Affected by the Snack Manufacturing Process

Instrumental colour measurements revealed that the unit operations applied in the snack manufacturing process had a significant effect, whereas the type of impregnation liquid employed was irrelevant.

As shown in Figure 1, the vacuum impregnation of apple slices caused, regardless of the impregnation solution used, a significant decrease in the value of the L* coordinate. The replacement of the air occluded in the porous structure of apples by the external liquid implied it changing from whitish and opaque to bright and translucent. After subsequent dehydration, all three-color coordinates rose markedly, most probably as a consequence of coloured compound concentration and the oxidation reactions taking place during the dehydration of vacuum-impregnated samples. The increase in a* and b* coordinates means a change from greenish to a reddish tone. As expected, enzymatic and non-

enzymatic browning reactions took place in a greater extent in air-dried than in freeze-dried samples, so the increase in the a* coordinate was significantly higher in the former ones. Furthermore, due to the longer exposure to the air required to achieve a lower water activity value (from 24 to 48 h), the rise in the a* coordinate was slightly higher in samples dehydrated to a lower water activity value (0.35). Water evaporation or sublimation taking place during the dehydration of vacuum impregnated samples explained the L* coordinate increase, with dehydrated apples becoming less translucent and opaquer than vacuum impregnated ones. The increase in the L* coordinate was greater in freeze-dried samples than in air-dried ones, thus suggesting that the water freezing, and further sublimation favoured a more porous structure than evaporation. On the contrary, air-drying might result in a more compact and less porous structure.

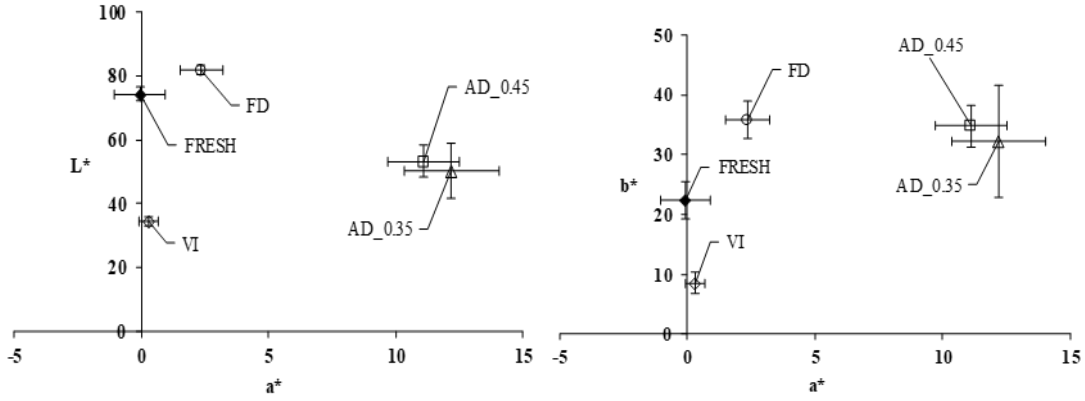

Figure 1. Colorimetric maps (L* vs. a* and b* vs. a*) of fresh apple (FRESH), vacuum-impregnated apple (VI), freeze-dried apple (FD) and air-dried apple at 40 °C until a final water activity of 0.45 (AD_0.45) or 0.35 (AD_0.35). Mean values of samples impregnated with the different impregnation liquids. Error bars represent the standard deviation of three replicates for each one of the different impregnation liquids.

3.4. Mechanical Properties as Affected by the Snack Manufacturing Process

Force (N) vs. distance (mm) curves obtained after the puncture tests are shown in Figure 2. Since the multifactor ANOVA (p-value < 0.05) revealed no significant effect of the impregnation liquid on the maximum force and the distance to breakage values, only curves of those samples impregnated with liquid 0% _0MPa are presented as an example. As can be observed, values of both mechanical properties analysed increased with the dehydration of vacuum impregnated apple slices, air-drying giving rise to significantly harder and more deformable snacks than freeze-drying. Furthermore, increasing the air-drying length in order to achieve lower water activity values (AD_0.35) caused a significant increase in the maximum force needed to break the sample, while slightly affecting the distance travelled by the punch. These results confirm that air-drying favours a rather rubbery and less crispy (crunchy/brittle) texture than freeze-drying. Indeed, the sublimation of water in freeze-dried samples resulted in the formation of a porous structure, whereas the evaporation of water during convective drying resulted in greater volume changes and structure collapse.

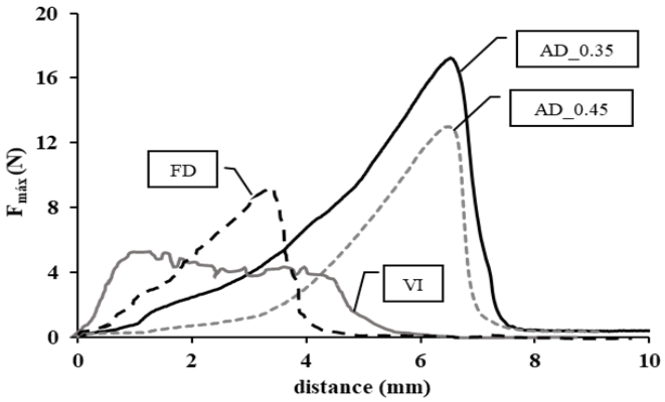

Figure 2. Typical force (in N) vs. distance (in mm) curves obtained after the puncture tests for apple samples impregnated with liquid 0% _0MPa (VI) and further freeze-dried apple (FD) or air-dried at 40 °C until a final water activity of 0.45 (AD_0.45) or 0.35 (AD_0.35).

As regards the shape of the curves, that of vacuum impregnated samples was very similar to that of fresh and porous plant tissues, made up of tightly packed and turgid cells. After a slight deformation, the vacuum impregnated tissue underwent multiple fractures as the punch advanced through it. In the case of dehydrated samples, the force increased rapidly with the distance after a slight (for FD samples) or pronounced (for AD samples) initial deformation.

3.5. Survival of *Lactobacillus salivarius* spp. salivarius during Snack Storage

Table 3 shows *Lactobacillus salivarius* spp. *salivarius* counts in dried apple samples after their storage for 7, 15 or 30 days under controlled conditions.

Table 3. Microbial counts in apple snacks during their storage for 30 days under controlled conditions. FD stands for freeze-dried apples, AD_0.45 stands for apples air-dried until reaching a aw ≈ 0.45 and AD_0.35 stands for apples air-dried until reaching a aw ≈ 0.35. Mean value of three replicates ± standard deviation. Different letters in the same column indicate statistically significant differences with a 95% confidence level (p-value < 0.05).

SAMPLE	TRE%_HPH	*Storage time*			*Log reduction*
		7 Days	*15 Days*	*30 Days*	
FD a_w = 0.25 ± 0.02	0%_0MPa	7.80 ± 0.05^{c}	7.66 ± 0.11^{fg}	7.35 ± 0.06^{h}	-0.76 ± 0.06^{g}
	10%_0MPa	8.23 ± 0.06^{d}	7.84 ± 0.04^{g}	7.10 ± 0.10^{gh}	-1.61 ± 0.10^{de}
	0%_100MPa	7.75 ± 0.04^{c}	7.46 ± 0.12^{f}	6.73 ± 0.02^{fg}	-1.66 ± 0.02^{d}
	10%_100MPa	7.84 ± 0.02^{c}	7.76 ± 0.06^{g}	6.71 ± 0.11^{g}	-0.65 ± 0.11^{gh}
AD_0.45 a_w = 0.42 ± 0.02	0%_0MPa	5.24 ± 0.09^{a}	5.1 ± 0.7^{b}	4.1 ± 0.4^{bc}	-3.6 ± 0.2^{c}
	10%_0MPa	6.05 ± 0.04^{b}	5.7 ± 0.2^{d}	4.1 ± 0.4^{c}	-4.2 ± 0.4^{b}
	0%_100MPa	6.11 ± 0.02^{b}	5.4 ± 0.4^{cd}	3.27 ± 0.13^{a}	-4.95 ± 0.13^{a}
	10%_100MPa	5.2 ± 0.4^{a}	4.2 ± 0.4^{a}	3.46 ± 0.09^{ab}	-4.34 ± 0.09^{b}
AD_0.35 a_w = 0.36 ± 0.03	0%_0MPa	6.32 ± 0.07^{b}	6.261 ± 0.012^{e}	6.09 ± 0.14^{ef}	-1.21 ± 0.07^{f}
	10%_0MPa	5.72 ± 0.02^{a}	5.64 ± 0.10^{d}	5.24 ± 0.05^{d}	-0.53 ± 0.02^{gh}
	0%_100MPa	6.00 ± 0.08^{a}	5.889 ± 0.012^{e}	5.22 ± 0.07^{de}	-1.4 ± 0.4^{ef}
	10%_100MPa	5.83 ± 0.05^{b}	5.58 ± 0.05^{c}	5.18 ± 0.10^{d}	-0.52 ± 0.05^{h}

Although storage negatively affected microbial population in all cases, it was especially evident in those samples air dried at 40°C until reaching a water activity of 0.45 (AD_0.45). Within a storage time of one month, the microbial viability was on average

reduced 1.1 ± 0.5-log10 in FD samples, 0.9 ± 0.4-log10 in AD_0.35 samples and 4.3 ± 0.5-log10 in AD_0.45 samples. These results suggest that, beyond structural changes taking place during the dehydration step, the water activity of the final product exerts a decisive role in the survival of the microorganism during its subsequent storage. From our results, probiotic survival during storage was particularly affected when the food matrix had a water activity around 0.42 ± 0.02, which is well above the value of 0.25 reported to ensure the minimum loss of viability in dried products [13,41]. To explain the high survival of *Lactobacillus salivarius* spp. *salivarius* during storage in AD_0.35 samples, one could refer to the water activity value closer to the optimum or to the production of heat-shock proteins observed in many *Lactobacillus* strains submitted to sub-lethal heat and oxidative stress [42].

Regarding the effect of trehalose addition and/or the homogenization at 100 MPa on the CECT 4063 strain's survival during storage, it was significantly affected by the water activity reached by the product at the end of the dehydration step. Survival of *Lactobacillus salivarius* spp. *salivarius* in both FD and AD_0.45 samples after 30 days of storage was not affected by the addition of trehalose and/or the homogenization. In contrast, cell survival in AD_0.35 samples improved significantly (p-value < 0.05) by including trehalose in the impregnation liquid composition, regardless of the application of a subsequent homogenization step (liquids 10%_0MPa and 10%_100MPa). Again, the production of heat-shock proteins by *Lactobacillus salivarius* spp. *salivarius* during long drying might be positively affected by sub-lethal osmotic stress induced when trehalose was added to the growing media. This hypothesis is reinforced by the synthesis of heat-sock proteins induced in *Lactobacillus delbrueckii* subsp. *bulgaricus* by acidic conditions [43] or in *Lactococcus lactis* by salt stress [44], which indicate that these proteins' synthesis is a more general stress response, rather than just a thermal stress response. In all cases, the impregnation with the juice subjected to homogenization at 100 MPa after fermentation (liquid 0%_100MPa) resulted in the greatest loss of viability after 30 days of storage.

This could be due not only to cell disruption caused by the application of a pressure gradient, but also to the increase in the availability of food bioactive compounds with antimicrobial properties [45].

3.6. Antioxidant Properties Change during Snack Storage

The net change in the antioxidant properties of the different probiotic snacks after 30 days of storage are shown in Figure 3.

The total phenol content increased 47% ± 2% in the case of freeze-dried samples (which had the highest counts at the end of the storage) but decreased in the case of air-dried ones. As previously reported for the content in *Lactobacillus salivarius* spp. *salivarius*, the loss of total phenols was significantly greater (p-value < 0.05) for those apple samples reaching a higher water activity at the end of the drying step (AD_0.45 samples). This direct relationship between the number of living cells and the content of total phenols was previously observed by Akman et al. [7] in samples impregnated with

Lactobacillus paracasei and suggests that probiotic cells that were efficiently attached to the apple structure protected phenolic compounds from degradation caused by the attack of reactive oxygen species or by the polymerization of the monomeric phenolic compounds [46].

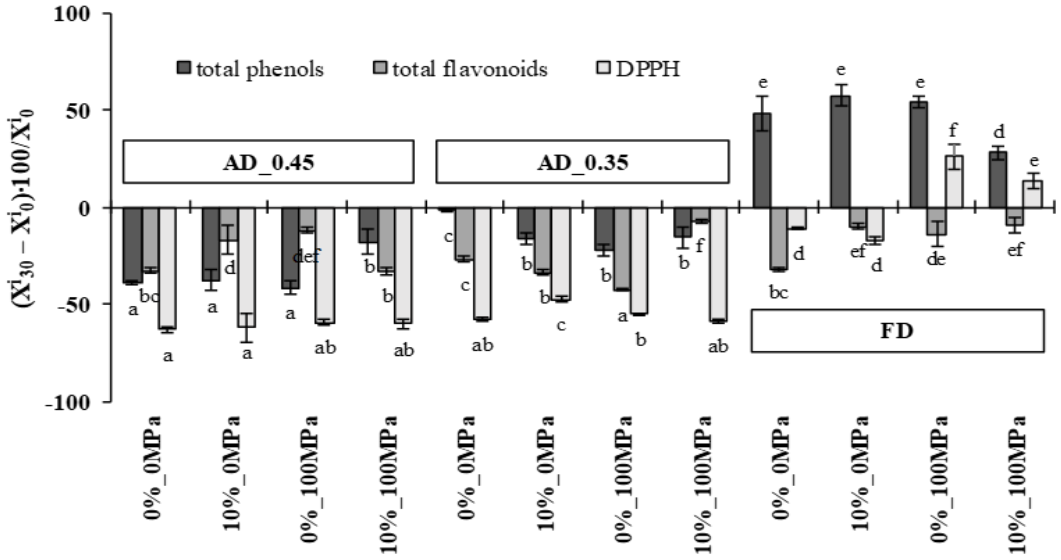

Figure 3. Antioxidant property change of apple snacks after 30 days of storage under controlled conditions. Xi0 and Xi30 respectively stand for the mass fraction in dry basis of component at the beginning and at the end of the storage, VI APP stands for vacuum-impregnated apples, FD stands for freeze-dried apples, AD_0.45 stands for apples air-dried until reaching a aw ≈ 0.45 and AD_0.35 stands for apples air-dried until reaching a aw ≈ 0.35. Error bars represent the standard deviation of three replicates for each one of the different impregnation liquids. Different letters in the same series indicate statistically significant differences with a 95% confidence level (p-value < 0.05).

Total flavonoid content decreased in all cases, but to a different extent, depending on the composition of the vacuum impregnation liquid, the dehydration treatment applied and the interaction between both factors. Total flavonoid loss in samples impregnated with liquid 0%_0MPa was, regardless of the dehydration treatment applied, around 30% ± 3%. Adding 10% of trehalose to the impregnation liquid formulation or homogenizing it at 100 MPa significantly reduced the total flavonoid loss to 13% ± 3% in both FD and AD_0.45, but slightly increased it to 38% ± 6% in AD_0.35 samples. Combining both variables in liquid 10%_100MPa resulted in a flavonoid loss like that obtained for samples impregnated with liquid 0%_0MPa after one-month storage of AD_0.45 samples but reduced it to 8.2% ± 1.5% in the case of FD and AD_0.35 samples.

Since the antioxidant activity is closely correlated with the presence of phenols and flavonoids [47], the DPPH radical scavenging activity also decreased in most of the cases after one month in storage. As stated previously for total phenols, the decrease in DPPH radical scavenging activity after storage was minimal for FD samples and maximal for AD_0.45 samples. Regarding the composition of the impregnation liquid, it was

especially evident when storing FD apples, in which the juice homogenization at 100 MPa involved a significant increase in DPPH radical scavenging activity.

4. Conclusions

All the stabilization techniques applied in this study had a negative impact on *Lactobacillus salivarius* CECT 4063 counts, but generally improved the antioxidant properties of apple samples. Freeze-drying, especially after adding 100 mg/g of trehalose to the growth medium, proved to cause the lowest decrease in the microbial population. Meanwhile, drying with air at 40°C up to a water activity of 0.45, especially when trehalose was not added to the growth medium and/or the vacuum impregnating liquid containing the microorganism was not homogenized, resulted in the greatest increase in both total phenol content (including flavonoids) and total ability to scavenge the DPPH$^{\bullet}$ radical.

Regarding the storage stability of *Lactobacillus salivarius* CECT 4063 in apple snacks, it was found to depend not only on water activity, but also on structural changes and/or heat-shock protein production induced by the different stabilization techniques applied. Therefore, the microbial count reduction within one month of storage was found to be higher in freeze-dried samples having the lowest water activity than in samples dried with air at 40°C up to a water activity of 0.35. Moreover, cell survival in the latter case improved significantly (p-value < 0.05) by adding trehalose to the impregnation liquid composition.

As regards the antioxidant compounds, only the loss of total phenols within one month of storage was observed to increase significantly (p-value < 0.05) with the water activity of the apple snacks, whereas the loss of total phenols and total ability to scavenge the DPPH$^{\bullet}$ radical were more affected by the composition of the vacuum impregnation liquid, the dehydration treatment applied and the interaction between both factors. In any case, antioxidant compounds prove to have the maximum stability during the storage of freeze-dried samples.

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ARTÍCULO 4

ANTIOXIDANTS BIOACCESSIBILITY AND *LACTOBACILLUS SALIVARIUS* (CECT 4063) SURVIVAL FOLLOWING THE *IN VITRO* DIGESTION OF VACUUM IMPREGNATED APPLE SLICES: EFFECT OF THE DRYING TECHNIQUE, THE ADDITION OF TREHALOSE AND HIGH -PRESSURE HOMOGENIZATION

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Abstract: To benefit the health of consumers, bioactive compounds must reach an adequate con-centration concentration at the end of the digestive process. This involves both an effective release from the food matrix where they are contained and a high resistance to exposure to gastrointestinal conditions. Accordingly, this study evaluates the impact of trehalose addition (10% w/w) and homogenization (100 MPa), together with the structural changes induced in vacuum impregnated apple slices (VI) by air-drying (AD) and freeze-drying (FD), on *Lactobacillus salivarius* spp. *salivarius* (CECT 4063) survival and the bioaccessibility of antioxidants during in vitro digestion. Vacuum impregnated apple slices conferred maximum protection to the lactobacillus strain during its passage through the gastrointestinal tract, whereas drying with air reduced the final content of the living cells to values below 10 cfu/g. The bioaccessibility of antioxidants also reached the highest values in the VI samples, in which the release of both the total phenols and total flavonoids to the liquid phase increased with in vitro digestion. The addition of trehalose and homogenization at 100 MPa increased the total bioaccessibility of antioxidants in FD and AD apples and the total bioaccessibility of flavonoids in the VI samples. Homogenizing at 100 MPa also increased the survival of L. salivarius during in vitro digestion in FD samples.

Keywords: apple structure; *Lactobacillus salivarius* spp. *salivarius* (CECT 4063); trehalose; high pressure homogenization; *in vitro* digestion; antioxidants bioaccessibility; air-drying; freeze-drying

1. Introduction

Health and wellness are trends that have conditioned the development of the food and beverage industry in the last decades [1]. Concern about boosting the immune system as a way to prevent non-communicable diseases and provide protection from pathogenic viral infections has grown considerably since the COVID-19 pandemic hit the world. As a result of the growing inclination of consumers towards preventive healthcare, the global functional food and beverage market size was valued at USD 258.80 billion in 2020 and is projected to double by 2028 [2]. In relation to ingredients, probiotics held the major share, which is mainly attributed to an increasing awareness regarding their prophylactic and therapeutic potential [3].

The functionality of probiotics is mainly based on bacterial interaction with host gut microbiota through a number of actions including increasing the production of vitamins, antioxidants, and short-chain fatty acids, modifying the intestinal microbiota, reducing the intestinal pH, or improving the intestinal barrier selectivity through higher mucin, immunoglobulin A, and defensins production [4]. Probiotics not only protect humans against gastrointestinal pathogens [5], but they may also alleviate gastrointestinal dysbiosis, lower serum cholesterol, ameliorate cancer and lactose intolerance, and prevent allergic and autoimmune disorders [6].

Due to their power to remedy both systematic metabolic diseases and genetic neurodegenerative disorders, probiotics are considered as the twenty-first century panpharmacon [3]. However, in order to achieve these health benefits, probiotics must be consumed regularly and reach the intestine as a viable strain in appropriate quantities (10^8-10^9 CFU/day) [7]. Therefore, probiotics used in food formulations must not only survive to the processing and storage stages, but they must also survive the harsh conditions of the gastrointestinal tract, such as the low pH of the stomach, and the digestive enzymes and bile salts of the small intestine [8]. Therefore, various methods have been proposed to enhance the viability of probiotic bacteria, such as strain and food carrier selection, the addition of prebiotics, cell immobilization, and microencapsulation or the induction of cellular stress-tolerance pathways [9].

With regard to the food matrix, research has mainly focused on the role played by its composition, which is often modified by adding ingredients that act as probiotic growth promoters (e.g., sugars, vitamins, minerals, prebiotics) or protectants (e.g., skim milk powder, whey protein, glycerol, lactose, fat, trehalose) [10]. Although dairy foods are the foods with the greatest potential as probiotic carriers, apples have also been demonstrated to be a suitable alternative [11,12] since they contain polyphenols (dihydrochalcones, flavanols, hydroxycinnamates, and flavanol), vitamins, minerals, lipids, peptides, and carbohydrates [13], in addition to fibers (cellulose, hemicellulose, and pectin) [14], that act as prebiotics. Phenolic compounds in apples are also responsible for the health-protecting effects related to apple consumption [15] due to their antioxidant properties and their capacity to neutralize free radicals and to protect cells against oxidative stress [16,17]. Furthermore, as it was recently reported by the authors of [18] for raw and fried tomato puree inoculated with *L. reuteri*, antioxidants may enhance the viability of probiotics during digestion. This makes some unit operations, such as high-pressure homogenization (HPH), particularly relevant to enhance probiotic survival under adverse conditions either in response to induced stress [19,20] or to the higher release of certain bioactives [21].

Denser, more viscous, or complex matrices have been reported to better protect probiotics during digestion, although they also limit their release [10]. However, studies which focus particularly on food structures are scarce and are based on cheese networks, in which it is easier to modify the structure without significantly varying the composition. Changes in the food structure are in most cases promoted by different processing techniques (e.g., boiling, roasting, frying, freezing, air-drying, freeze-drying). In particular, freeze-drying results in high-value products with advanced rehydration properties, a high porosity, and limited shrinkage which, due to the low processing temperatures, preserves their nutrients, colour, aroma, and flavours [22]. On the contrary, convective dried products tend to have a low porosity, a high apparent density, poor rehydration properties and, due to high temperatures and long processing times, they lose a large amount of nutrients, flavour, and aroma compounds.

For all the aforementioned reasons, this study aims to evaluate the effect that the stabilization of vacuum impregnated apple slices by convective drying and freeze-drying has on *Lactobacillus salivarius* spp. *salivarius* (CECT 4063) survival and on the bioaccessibility of antioxidants (total phenols, total flavonoids, and total ABTS and DPPH scavenging compounds) during an in vitro simulation of gastrointestinal digestion. Simultaneously, information about the impact of trehalose additions to the snack formulation and of the homogenization at 100 MPa of the liquid containing the probiotic are also gathered.

2. Materials and Methods

2.1. Microbial Strain and Raw Materials

The selection of both the microbial strain and the raw materials was based on our previous research of probiotic apple snacks with the potential ability to treat and prevent Helicobacter pylori infection [23-26]. Therefore, the *Lactobacillus salivarius* spp. *salivarius* CECT 4063 strain was used as a bacterial culture. The lyophilized strain was supplied by the Spanish Type Culture Collection of Paterna (Valencia, Spain). Following the manufacturer's instructions, the microbial strain was first reactivated in sterile MRS broth (Scharlau Chemie®, Barcelona, Spain) at 37 °C for 24 h and then transferred to commercial clementine juice (Hacendado brand), which was used as a probiotic carrier. Finally, apples (var. *Granny Smith*) cut into 5 mm slices (20 mm internal diameter and 65 mm outer diameter) were used to host the probiotic. The selection of this solid matrix was based on its high homogeneity and porosity compared to other fruits [27].

2.2. Sample Preparation

Different food matrices used in this study were manufactured according to the procedure described in Burca-Busaga et al. [26]. On the one hand, vacuum impregnated samples (VI apples) were obtained in a VT 6130M Heraeus Vacutherm Oven (Thermo Scientific) connected to a LVS 210T laboratory vacuum system (Welch Ilmvac™, Fisher Scientific, Madrid, Spain) by the immersion of the apple slices in the corresponding impregnation liquid in a 1:5 (w/v) ratio, the application of a vacuum pressure of 50 mbar for 10 min, and the restoration of the atmospheric pressure, which was maintained for another 10 min. On the other hand, air-dried samples (AD apples) were obtained by drying VI apples in a CLW 750 TOP+ tray dryer (Pol-Eko-Aparatura SP.J.) with a cross flow of air at 2 m/s and 40 °C up to a water activity value of 0.35. Finally, freeze-dried samples (FD apples) were obtained by keeping VI apples at −40 °C for 24 h in a Matek CVN-40/105 ultra-freezer and the further sublimation of the frozen water at −45 °C and 0.1 mbar for 24 h in a Telstar Lioalfa-6 freeze-drier.

As the impregnation liquid, commercial clementine juice (Hacendado brand) containing 9.8 g/L of NaHCO3 (Sigma-Aldrich, Madrid, Spain) and 5 g/L of yeast extract (Scharlau Chemie®, Barcelona, Spain), inoculated with $1.4 \pm 0.3 \times 10^6$ CFU/mL of *L. salivarius* and then incubated at 37 °C for 24 h was used (liquid 0%_0MPa). In certain experiments, 100 g/kg of food-grade trehalose from tapioca starch (TREHATM, Cargill Ibérica) was added to the juice prior to inoculation (liquid 10%_0MPa). In other experiments, the fermented liquid was homogenized at 100 MPa in a laboratory scale high pressure homogenizer (Panda Plus 2000, GEA-Niro Soavi) before it was used as an impregnation liquid (liquid 0%_100MPa). Based on previous findings [28], homogenization at 100 MPa was not applied to the liquid containing 10% (w/w) of trehalose since it significantly reduced (p-

value < 0.05) the survival of *L. salivarius* following the in vitro digestion of the inoculated clementine juice. Final counts in any of the vacuum impregnation liquids were in the order of 6 ± 2 × 10^8 CFU/mL.

2.3. *In Vitro* Simulation of Gastrointestinal Digestion

The *in vitro* simulation of gastrointestinal digestion of the different food matrices was performed in sterile conditions following the protocol described by García-Hernández et al. [18], with some modifications. Each sample was digested three times in duplicate for the determination of both the microbial and the antioxidant properties.

For the oral stage simulation, apple samples were manually cut into pieces of about 2 × 2 × 5 mm^3 and mixed with human saliva in a ratio 1:1 (w/v) and 1:2 (w/v), for non-dehydrated (VI apples) and dehydrated samples (both AD and FD apples), respectively. After grinding with a T 25 digital ULTRA-TURRAX® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 8500-9500 rpm for 1 min, the simulated oral bolus was mixed in a ratio 1:5 (w/v) with a 3 g/L solution of 3200–4500 U/mg pepsin porcine (Sigma-Aldrich, Madrid, Spain) in a sterile saline solution at 0.5% (w/v) adjusted to pH 2 with HCl (0.5 N). Following 2 h of constant agitation at 100 rpm and 37°C on an orbital shaker (Optic Ivymen Systems TM), the resulting simulated chime was mixed in a ratio 1:1.8 (w/w) with a 1 g/L solution of 8 × USP porcine pancreatin (Sigma-Aldrich, Madrid, Spain) in a sterile saline solution at 0.5% (w/v) adjusted to pH 8 with NaOH (0.1 N). This mixture remained for another 4 h under constant agitation at 100 rpm and 37 °C on an orbital shaker (Optic Ivymen Systems TM).

Sampling for the microbial counts was performed at the end of the oral phase at different times along gastric digestion (0, 10, 30, 60, and 120 min) and at different times along intestinal digestion (0, 30, 60, 120, 180, and 240 min). For each time point, 1 mL of the liquid phase was withdrawn from the reaction vessel. Sampling for the antioxidant properties was only performed at the end of both the gastric and the intestinal phases by separating 1 mL aliquots from the corresponding liquid phase.

2.4. Analytical Determinations

2.4.1. Moisture Content and Water Activity

The moisture content was obtained from the weight loss undergone by a certain amount of the sample when dried in a vacuum oven (Vaciotem-T, J.P. Selecta) at 60°C and 200 mbar until a constant weight was reached. The water activity was measured in a CX-2 AquaLab dew point hygrometer (Decagon Devices, Inc., Pullman, WA, USA) at 25°C.

2.4.2. Microbial counts

Colony counts were measured by serial decimal dilution in sterile distilled water and plated on MRS Agar (Scharlau Chemie®, Barcelona, Spain). Since the microbial growth did not significantly increase under anaerobic conditions, the plates were incubated in aerobiosis at 37°C for 24 h. The survival of the microbial strain during each stage of *in vitro* gastrointestinal digestion was then calculated as the ratio between the microbial concentration at the end of the stage and the microbial concentration at the end of the previous one, both referred to the same basis. Likewise, probiotic survival during the entire *in vitro* gastrointestinal digestion was calculated as the ratio between the microbial concentration in the liquid phase at the end of the intestinal stage and the

microbial concentration of the undigested sample. All survival results were expressed as a percentage.

2.4.3. Antioxidant properties

Measurements in the case of the undigested samples were carried out on the supernatants resulting from the mixing of 2 g of the VI apple (which was reduced to 0.35 g in the case of AD and FD apples) with 20 mL of an 80:20 (v/v) methanol in a water solution, dispersing for 2 min at 5000 rpm with a T 25 digital ULTRA-TURRAX® disperser (IKA®-Werke GmbH & Co. KG, Staufen, Germany), shaking in the dark for 1 h at 200 rpm on an orbital shaker (Rotabit, J.P. Selecta, Barcelona, Spain) and subsequent centrifugation at 10,000 rpm and 4 °C in a Medifriger-BL-S (J.P. Selecta, Barcelona, Spain) centrifuge. Measurements at the end of both the gastric and the intestinal stage were directly carried out on the resulting liquid phases, which were diluted with distilled water in a 1:5 (v/v) ratio for AD and FD apples.

The total phenol content, total flavonoid content, and the overall antioxidant activity of the target samples were spectrophotometrically determined following the procedures described in Burca-Busaga et al. [26].

The total phenol content, which was obtained from the blue colour intensity that results after the reaction between the Folin–Ciocalteu reagent (Sigma-Aldrich, Madrid, Spain) and the phenolic compounds present in the sample, was measured at 760 nm, and expressed as mg of the gallic acid equivalents per gram of the dried sample (mg GAE/g dw). Specifically, 125 µL of the sample, 125 µL of the Folin–Ciocalteu reagent, and 500 µL of double distilled water were mixed. Following 6 min of reaction in the dark, 1.25 mL of 7.5% (w/v) sodium bicarbonate and 1 mL of redistilled water were added and left to react for another 90 min.

The total flavonoid content, which was assessed following the aluminium chloride method, was measured at 368 nm, and expressed as mg of the quercetin equivalents per gram of the dried sample (mg QE/g dw). For that purpose, 1.5 mL of sample reacted for 10 min with 1.5 mL of a 2% (w/v) solution of aluminium chloride in methanol.

Finally, the antioxidant activity was measured by the DPPH method, which consists of measuring the colour change at 515 nm from deep-violet to pale-yellow undergone by radical 1,1-diphenyl-2-picrylhydrazyl when reduced by antioxidants or other radical species and expressed as mg of the Trolox equivalents per gram of the dried sample (mg TE/g dw). In this case, 100 µL of the sample, 900 µL of methanol, and 2000 µL of a 100 mM DPPH solution in methanol (v/v) reacted for 30 min in a spectrophotometry cuvette. A blank was used in which the sample was replaced by the same volume of redistilled water.

The bioaccessibility of each compound, defined as the portion that is released from the food matrix into the gastrointestinal tract and thus becomes available for intestinal absorption [17], was obtained as the ratio from its concentration in the liquid phase at the end of the intestinal stage and the concentration in the sample before digestion.

2.5. Statistical Analysis

The statistical analysis of the experimental data was performed by means of a one-way or multifactorial ANOVA (confidence level of 95%) carried out with the Statgraphics Centurion XVI tool.

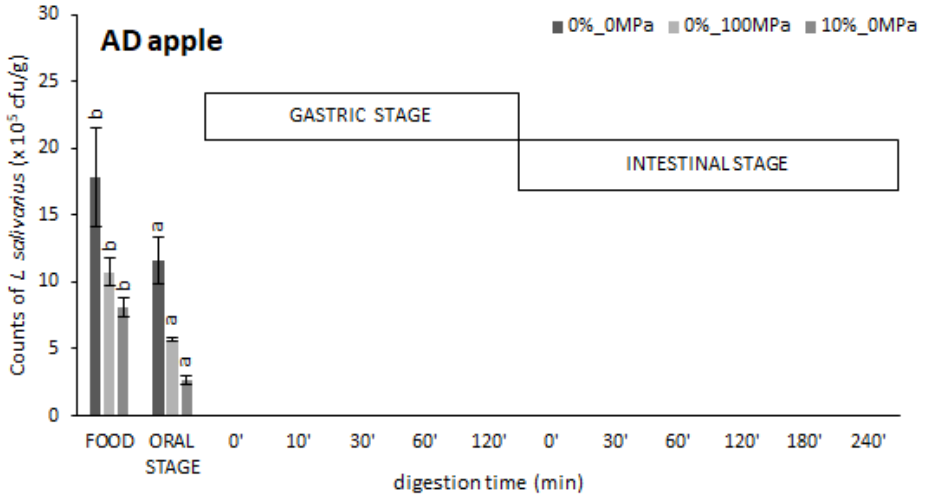
3. Results and Discussion

3.1. Effect of the Food Matrix and the Vacuum Impregnation Solution on Probiotic Survival during *In Vitro* Digestion

The effect of the food matrix (VI, AD, or FD apples) and the vacuum impregnation liquid (0%_0MPa, 0%_100MPa, or 10%_0MPa) on *L. salivarius* counts along the in vitro simulation of the gastrointestinal human digestion were recorded and are shown in Figure 1. The values obtained for the undigested samples (FOOD) were of the same order as those reported in a previous study by Burca-Busaga et al. [26].

Viable counts in both the VI and FD apples were hardly affected by the exposure to simulated mouth conditions. However, those in the AD apples decreased by 34 ± 4%, 47 ± 6%, and 68.1 ± 1.2% for liquids 0%_0MPa, 0%_100MPa, and 10%_0MPa, respectively.

Given that the microbial counts along the *in vitro* digestion were performed on the liquid phase, it is postulated that the microorganisms were completely released from the solid matrix to the salivary fluid in the VI and FD apples, but only partly in the AD samples. Leaching has previously been reported to be fast and excessive for FD apple cubes and a bit slower for AD apple cubes since freeze-drying causes a more extensive cell wall rupture than the convective process [29]. As for the significantly lower release of *L. salivarius* from the AD apples that were impregnated with the liquid that included 10% of trehalose in its composition, this is consistent with the ability of this disaccharide to replace the water of hydration at the membrane-fluid interface, thus preventing structural collapse as the tissue is dried [30].



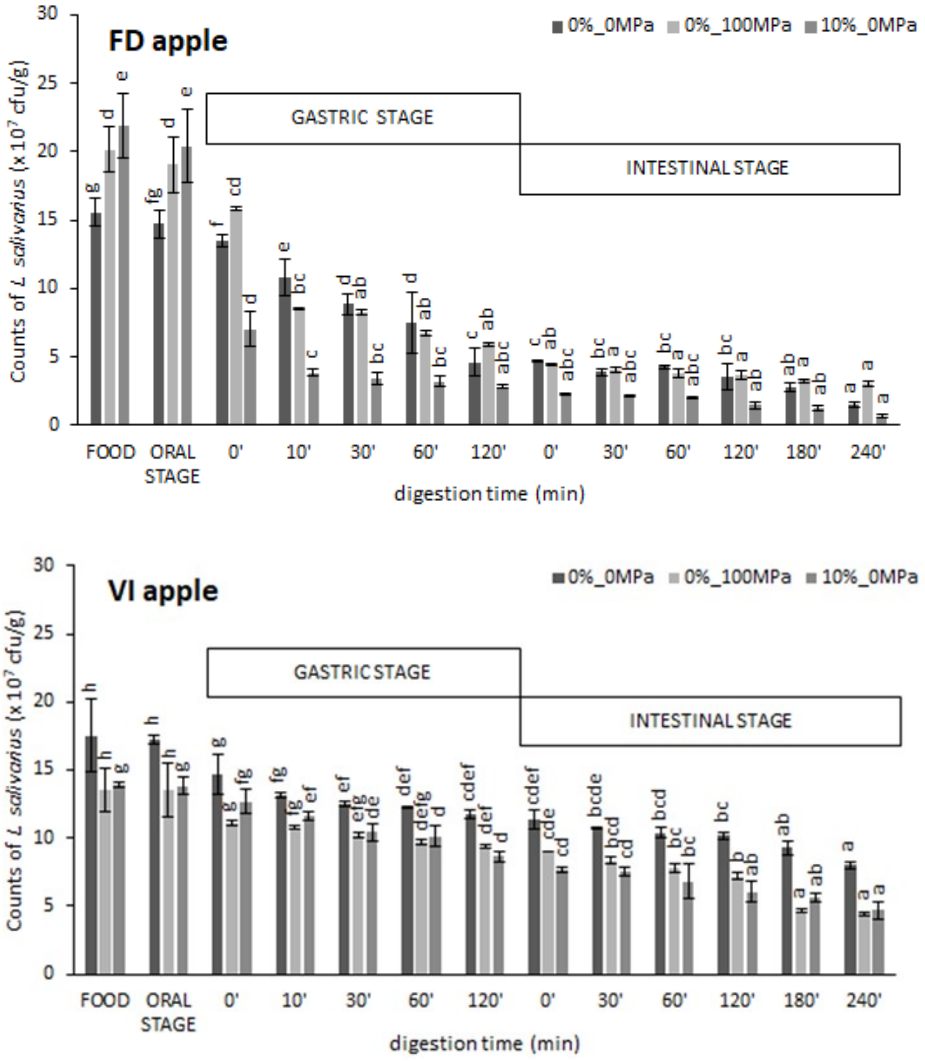


Figure 1. Microbial counts of *L. salivarius* along the gastrointestinal *in vitro* digestion of different food matrices (VI: vacuum impregnated apples; FD: freeze-dried apples; AD: air-dried apples) made with each of the impregnating solutions. Error bars represent the standard deviation of triplicates from two independent treatments. $^{abc...}$ different letters within the same series indicate statistically significant differences (p-value < 0.05).

A progressive decline in the microbial counts was observed in most cases along the second digestive step. These expected results are attributed to the ability of pepsin to destroy the peptide bond between amino acids and to degrade the microbial cell membrane [31]. However, as pointed out by García-Hernández et al. [18], microbial death due to the shock produced by the gastric juices is more likely to occur in those bacteria whose cell wall had been previously damaged by external factors. In accordance with this, a reduction in the viable number after the gastric stage was minimum in the VI apples (~ 38 ± 8% on average for samples impregnated with liquids 0%_0MPa, 0%_100 MPa, and 10%_0MPa) and increased with the application of a dehydration step (Table 1). Of the two dehydration techniques, freeze-drying was the one with the least impact on the microbial viability (a survival of 21 ± 8% on average for the samples impregnated with liquids 0%_0MPa, 0%_100 MPa, and 10%_0MPa). In the case of AD apples, the only contact with the simulated gastric juice reduced the number of viable cells to less than 10 cfu/g. As regards the vacuum impregnation liquid (Table 1), adding 10% of trehalose to its composition slightly reduced the survival of *L. salivarius* during *in vitro* gastric

digestion possibly due to induced osmotic stress. On the contrary, homogenizing the microorganism at 100 MPa had no effect on its survival in VI apples after 120 min of exposure to the simulated gastric juice, but significantly increased that in FD apples. It seems that those cells that survived the freeze-drying step after their homogenization at 100 MPa were better prepared to withstand adverse stomach conditions. In a previous study [28], the survival of *L. salivarius* in gastric simulated conditions after the *in vitro* digestion of the vacuum impregnation liquids was reported to be 36 ± 7%, 57 ± 3%, and 51.34 ± 0.13% for liquids 0%_0MPa, 0%_100MPa, and 10%_0MPa, respectively. Significantly higher values obtained in the present study after the *in vitro* digestion of VI apples (63 ± 7% on average) confirms that including the lactobacillus into the porous structure of apple slices protects it as it passes through the upper gastrointestinal tract. In particular, the grip of microorganisms to apples mainly occurs in the intercellular spaces of the parenchymal tissue of the fruit [14]. This finding is particularly interesting in relation to the ability of *L. salivarius* to inhibit pro-inflammatory cytokine secretion from *Helicobacter pylori* (a well-known gastric pathogen) infected cells [32].

Exposure to the small intestinal conditions (pancreatin solution at pH 8.0) resulted in further changes in the survival rate of *L. salivarius*. Again, the loss of viability was significantly higher (p-value < 0.05) in FD apples than in VI apples, and while homogenization at 100 MPa slightly increased *L. salivarius* survival in the simulated intestinal juice digestion in FD samples, it significantly reduced it in VI ones (p-value < 0.05). Adding 10% of trehalose to the vacuum impregnation liquid negatively affected the survival of lactobacillus during intestinal digestion in both VI and FD apples, but this was much greater in the case of the FD samples in which the disaccharide reached a higher concentration. Given that extracellular trehalose cannot provide sufficient protection for cells during dehydration and gastrointestinal digestion [33], the trehalose added to the growing media was neither imported nor did it induce the expression of trehalose-synthesizing genes to a sufficient extent.

The survival of *L. salivarius* during the entire gastrointestinal digestion was obtained, as explained in Section 2.4.2, from its survival during the simulated oral, gastric, and intestinal stages. As shown in Table 1, the survival of *L. salivarius* in VI apples after the simulated gastrointestinal digestion was similar to that previously reported by Barrera et al. [28] for vacuum impregnation liquids (between 26 ± 5% and 33 ± 2% for liquids 0%_0MPa and 0%_100 MPa, respectively), but was significantly higher than that obtained for *L. salivarius* in FD apples or in MRS Broth. These differences, however, did not prevent the microorganism from reaching the end of the digestive process in a sufficient concentration (>10^7 cfu/g) to exert a beneficial effect on the consumer's health. The survival of *L. salivarius* during the simulated gastrointestinal digestion of both VI and FD samples was significantly higher (~0.4 log reduction and ~1 log reduction, respectively) than that reported by Valerio et al. (2020) for *L. paracasei* IMPC2.1 in a pectin-coated dehydrated apple snack containing ≥ 9 log cfu/20 g portion (~2 log reduction). On the same microbial strain, air drying conducted at 60 °C for 1 h, 50 °C for 30 min, and 40 °C up to 24 h was less detrimental to its survival during the digestion process than one-stage drying at 40 °C [25]; on the contrary, the encapsulation of the lactobacillus by HPH at 70 MPa considerably reduced the survival of the strain during the digestion process in FD apple slices.

Table 1. Microbial concentration of the digested samples (final counts) and the survival percentage of *L. salivarius* to each stage (oral, gastric, and intestinal) and the entire simulated digestion (total) as affected by the food matrix and the growing media. Mean value ± standard deviation of triplicates from two independent treatments.

Treatment		Oral Stage $\frac{X_{OS}^{lb}}{X_{UF}^{lb}} \cdot 100$	Gastric Stage $\frac{X_{GS}^{lb}}{X_{OS}^{lb}} \cdot 100$	Intestinal Stage $\frac{X_{IS}^{lb}}{X_{GS}^{lb}} \cdot 100$	Total $\frac{X_{IS}^{lb}}{X_{UF}^{lb}} \cdot 100$	Final Counts (Log cfu/g)
MRS Broth		**-**	**33 ± 2 [b]**	**48.50 ± 0.13 [bc]**	**15.8 ± 1.4 [b]**	**8.566 ± 0.004 [f]**
VI apple	**0%_0MPa**	97 ± 4 [de]	68 ± 3 [d]	68 ± 8 [d]	47 ± 5 [d]	7.904 ± 0.012 [e]
	0%_100MPa	100 ± 2 [e]	65 ± 7 [d]	48 ± 2 [b]	31 ± 4 [c]	7.644 ± 0.015 [d]
	10%_0MPa	93 ± 7 [d]	55 ± 9 [c]	54 ± 6 [bc]	28 ± 5 [c]	7.67 ± 0.06 [d]
FD apple	**0%_0MPa**	99 ± 2 [de]	17 ± 5 [a]	53 ± 9 [bc]	9 ± 2 [a]	7.19 ± 0.05 [c]
	0%_100MPa	94 ± 4 [de]	28 ± 5 [b]	58 ± 5 [c]	16 ± 3 [b]	7.438 ± 0.012 [b]
	10%_0MPa	93 ± 2 [de]	14 ± 3 [a]	24 ± 3 [a]	3.1 ± 0.2 [a]	6.82 ± 0.07 [a]
AD apple	**0%_0MPa**	66 ± 4 [c]	n d	n d	n d	n d
	0%_100MPa	53 ± 6 [b]	n d	n d	n d	n d
	10%_0MPa	31.9 ± 1.2 [a]	n d	n d	n d	n d

[abcde] different superscripts in the same column indicate statistically significant differences (p-value < 0.05). X_{UF}^{lb} are the colony counts of undigested food (cfu/g dw); X_{OS}^{lb} are the colony counts at the end of the oral stage (cfu/g dw); X_{GS}^{lb} are the colony counts at the end of the gastric stage (cfu/g dw); and X_{IS}^{lb} are the colony counts at the end of the intestinal stage (cfu/g dw).

3.2. Effect of the Food Matrix and the Vacuum Impregnation Solution on the Bioaccessibility of Antioxidants during *In Vitro* Digestion

Total phenols (mg GAE/g dw), total flavonoids (mg QE/g dw), and overall antioxidant activity (mg TE/g dw) measured before and after each stage of the *in vitro* simulation of the gastrointestinal human digestion as affected by the food matrix (VI, AD, or FD apple) and the vacuum impregnation liquid (0%_0MPa, 0%_100MPa, or 10%_0MPa) are shown in Figure 2. In accordance with the previous findings of the research group [26], the values obtained for the undigested vacuum impregnated apples were 5.6 ± 0.4 mg GAE/g dw, 1.4 ± 0.4 mg QE/g dw, and 6.5 ± 0.5 mg TE/G dw, regardless of the composition of the vacuum impregnation liquid used. These values remained almost constant after freeze-drying, but significantly increased (p-value < 0.05) after convective drying.

Following the gastric phase of the *in vitro* digestion, a significant increase (p-value < 0.05) in the content of both the total phenols and flavonoids was observed for VI samples. On average, the content of the total phenols and flavonoids increased by 52 ± 11% and 152 ± 37%, respectively, after the action of the simulated gastric juice. This is in agreement with previous findings [34,35] and indicates that the 3 g/L pepsin solution adjusted to pH 2 allowed more glycosidic bonds to be broken and enabled the release of more phenolic compounds (especially of the flavonoid type) than the chemical extraction with an 80% (v/v) solution of methanol in water. More specifically, apple samples impregnated with liquid 0%_0MPa showed the greatest increase in total phenols (from 5.27 ± 0.07 to 8.7 ± 0.6 mg GAE/g dw) but the lowest increase in total flavonoids (from 1.78 ± 0.08 to 3.3 ± 0.5 mg QE/g dw). Unlike the phenolic and flavonoid contents, the

DPPH values of the VI apples were 40 ± 4% lower after the gastric phase of digestion, regardless of the composition of the VI liquid. Such a decrease in the antiradical activity could be attributed to the presence of other antioxidant compounds different from polyphenols that are less soluble in the gastric juice and/or more sensitive to the acidic conditions of the stomach. This could be the case with carotenoids which, owing to the numerous double bonds of their chemical structure, are particularly susceptible to oxidation in acidic media [35]. As an example, the overall recovery of carotenoids after the gastric phase of mandarin pulp *in vitro* digestion was around 79% [36], but it decreased to 36-63% when a blended fruit juice containing orange, pineapple, and kiwi was digested [35].

As to the dried apples, all three antioxidant properties in the samples subjected to gastric conditions were significantly lower (p-value < 0.05) than in the undigested ones. A decrease in the total phenols in both AD and FD apples was of the same order (36 ± 4%) and, although in total flavonoids it was slightly higher in FD (68 ± 4%) than in AD apples (59 ± 8%), a reduction in the overall antioxidant activity was significantly higher (p-value < 0.05) in AD (57 ± 5%) than in the FD apples (38 ± 15%). Since neither the total content of phenols or flavonoids in the VI apples were negatively affected by the gastric conditions, the decrease observed in both the AD and FD apples could be attributed to the differences in the food matrix between the dry and wet samples. It follows that the release of polyphenols from the solid matrix to the gastric fluid was negatively affected by the structural changes derived from dehydration, regardless of the specific technique used. However, as aforementioned for *L. salivarius*, the leaching of other antioxidant compounds might be more effective from FD samples which are known to suffer a more extensive cell wall rupture. Of all the dehydrated samples, those impregnated with liquid 0%_100 MPa and subsequently FD showed the lowest decline in DPPH values after the gastric stage of the *in vitro* digestion. This would confirm the application of high-pressure processing on plant foods as a useful tool to improve the extractability and bioaccessibility of antioxidant compounds either through producing changes in the membrane permeability and the disruption of cell walls and cell organelles (as reported by Fernández-Jalao, et al. [17] for “Golden Delicious” apples subjected to 400-600 MPa for 5 min) or through reducing the average particle size (as reported by Di Nunzio et al. [21] for mandarin juice homogenized at 20 MPa).

Following the intestinal phase of the *in vitro* digestion, the total phenol content of VI apples increased between 15% and 20%, depending on the composition of the vacuum impregnation liquid. However, that of AD and FD apple slices decreased by 11 ± 3%, regardless of the drying technique or the vacuum impregnation liquid used. Based on these findings, it could be said that the total polyphenol release that took place during the gastric digestion of VI apples was uncompleted since vacuum impregnation allows these bioactive compounds to be better retained and protected in the intercellular spaces. This result would be in accordance with that reported by Liu et al. [37], who found that the amount of total extractable phenols released from freeze-dried apple pomace powder increased significantly (from 4.4 to 17.5 mg GAE/g) from the gastric to the jejunal phase due to alkaline hydrolysis causing the breaking of the ester bond linking phenolic acids to the cell wall. However, since Chen et al. [38] also observed that the phenolic content of red delicious apple extracts after the duodenal phase of *in vitro* digestion was 2.45 times higher than that obtained after the gastric phase, some other changes in polyphenolic compounds such as an interaction with other dietary components, as well as the modification of the chemical structure or solubility might

happen. Interestingly, these same changes are considered responsible in other studies for the decrease in the total polyphenols observed in the mild alkaline intestinal conditions [39-41]. With regard to chlorogenic acid, the most abundant apple polyphenol, Bouayed et al. [40] reported that between 41% and 77% was degraded during the intestinal digestion of fresh apples, an amount that increased up to 100% in the case of flavanols and caffeic acid belonging to the group of hydroxycinnamic acids. In addition, isomers of chlorogenic acid, mainly crypto chlorogenic acid and neochlorogenic acid, were found to arise in the intestinal phase at concentrations almost comparable to that of residual chlorogenic acid. Nevertheless, the total polyphenolics in the intestinal medium was 40% lower than in the gastric medium. In a different study [41], the total polyphenol content of the air dried apple slices was reported to decrease from 121 ± 21 mg GAE/100 dw to 104 ± 25 mg GAE/100 dw (~14%) due to a pH change from acid (gastric digestion) to alkaline (intestinal digestion), and from 272 ± 8 mg GAE/100 dw to 188 ± 9 mg GAE/100 dw (~31%) when apple slices were enriched with grape juice by vacuum impregnation and ohmic heating at 50 °C before convective drying.

Regarding the total flavonoid content, it remained fairly stable after the intestinal phase simulation and only decreased by 7% on average, regardless of the food matrix and the vacuum impregnation liquid. Out of all the flavonoids in apples, epicatechin, procyanidin B2, and quercetin-3-O-galactoside were found to be the most degraded ones under the weak alkaline conditions of the intestinal digestion [37]. Of the three different matrices analysed, FD apples were the ones that showed a significantly higher loss of flavonoids (p-value < 0.05), while that in VI apples was found to be the lowest. As regards the vacuum impregnation liquid, the 0%_100 MPa was the one that best prevented the degradation of the flavonoids. A similar positive effect on the bioaccessibility of flavonoids as a result of the ortanique low pulp juice homogenization at 20 MPa was previously observed by Di Nunzio et al. [21] and was explained in terms of the reduction in the particle size of the juice that facilitates the release of bioactives from the matrix.

Compared with the gastric phase of digestion, the overall antioxidant activity of both the VI and AD samples increased significantly (p-value < 0.05), but particularly in VI apples when liquid 0%_0MPa was used as an impregnation solution, after the intestinal phase of digestion. On the contrary, the DPPH values of the FD samples decreased by 36 ± 4%, 21 ± 3%, and 28 ± 3% for vacuum impregnation liquids 0%_0MPa, 0%_100 MPa, and 10%_0MPa, respectively. An increase in the overall antioxidant activity of VI apples during the intestinal phase was consistent with the increase in the total phenol content. Likewise, a decline in the ability of FD apples to scavenge DPPH radicals matched with the decrease in both the total phenol and flavonoid content. However, the increase in the overall antioxidant activity of AD apples that took place during the intestinal phase despite the decrease in both the total phenol and flavonoid content suggests that novel compounds formed during the hot air-drying processing, such as Maillard-derived melanoidins responsible for browning during the drying process, might still be released to the soluble fraction under alkaline conditions.

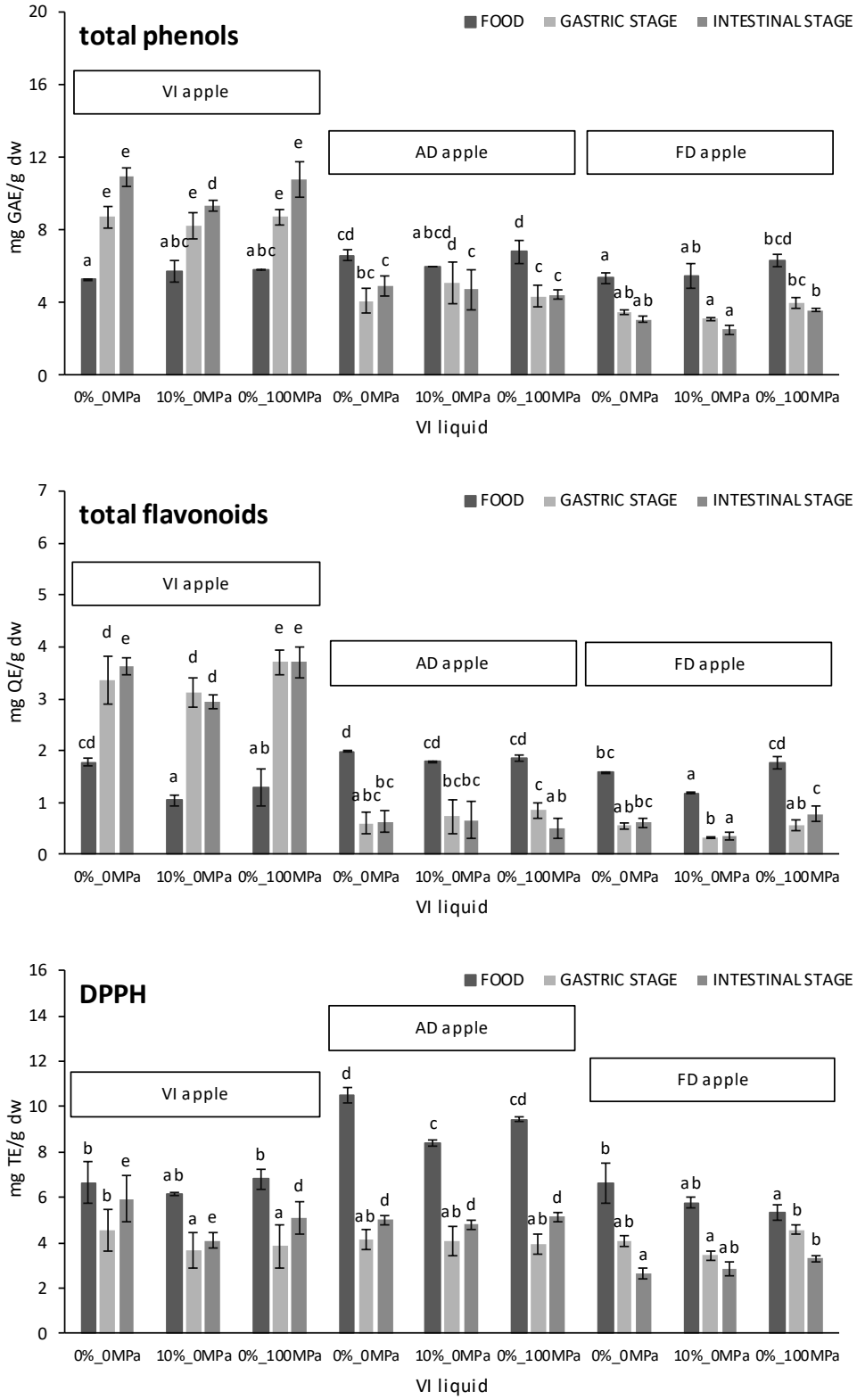


Figure 2. Antioxidant content at each stage of the gastrointestinal *in vitro* simulation (before digestion and after both the gastric and intestinal stages of digestion) as affected by the food matrix (VI, AD, and FD apples) and the vacuum impregnation liquid (0%_0MPa, 10%_0MPa, and 0%_100MPa). Error bars represent the standard deviation of triplicates from two independent treatments. $^{abc...}$ different letters within the same series indicate statistically significant differences (p-value < 0.05) among samples analysed at the same moment of the process.

All these changes affecting the antioxidant properties of the apple samples along the digestive process have a direct impact on their bioaccessibility, which is a prerequisite for their ability to be effectively absorbed from the intestinal tract into the blood circulation and delivered to the appropriate location within the body [42]. The bioaccessibility of the total phenols, the total flavonoids, and the total antioxidant activity as affected by the food matrix (VI, AD, or FD apples) and the vacuum impregnation liquid (0%_0MPa, 0%_100MPa, or 10%_0MPa) are shown in Figure 3. As it can be observed, the bioaccessibility of the phenolic compounds, including flavonoids, and of all the other antioxidant compounds was significantly higher (p-value < 0.05) for VI apples. When liquids 0%_100 MPa or 10%_0MPa were used as impregnating solutions, the total flavonoids released from VI to the liquid phase at the end of the intestinal stage increased 2.9-fold compared with their content in the undigested samples. This value was reduced to 2.0-fold for vacuum impregnation liquid 0%_0MPa. A totally opposite effect on the bioaccessibility of both the total phenols and the antioxidants from the VI apples was observed, so that samples impregnated with liquid 0%_0MPa showed the highest values. In any case, the bioaccessibility values obtained for the VI apples were considerably higher than expected; this could be related to the microbial activity of the lactobacillus strain. This is in line with the findings of Di Nunzio et al. [21], who reported a significant increase in narirutin and didymin bioaccessibility by adding 8 log cfu mL^{-1} of *L. salivarius* to ortanique juice homogenized at 20 MPa. As was also argued by these authors, this result can be attributed to the ability of the microorganism to enhance the release of phenolic compounds linked to fibers and other components of the food matrix. The total phenolic content, anthocyanin, and the DPPH radical scavenging capacity of Sohiong juice were also reported to increase in the presence of *L. plantarum* [43], possibly due to its β-glucosidase activity, as well as to its ability to biotransform the bioactive compounds into their metabolites or to chelate metal ions and to scavenge reactive oxygen species.

The bioaccessibility values obtained for both the AD and FD apples were of the same order: between 46.5% and 78.9% for total phenols, between 27.5% and 44.5% for total flavonoids, and between 39.7 and 61.2% for DPPH scavenging potential. In comparison, Bouayed et al. [40] found that the bioaccessibility of phenolic compounds in apples was 55%, which is in the range of the values obtained in the present study, and Gullon et al. [42] found that the bioaccessibility of polyphenolic compounds present in apple bagasse flour was 91.58%, which is much higher than the values obtained in the present study. Only significant differences (p value < 0.05) were found between AD and FD apples for the bioaccessibility of the total phenols and the total antioxidant values and when liquid 10%_0MPa was used as the impregnating solution, with AD apples showing higher values than FD ones. Regarding the vacuum impregnation liquid, it hardly affected the bioaccessibility of both the total phenols and flavonoids, but it did affect that of the total antioxidants. In general terms, both the addition of trehalose and the homogenization at 100 MPa increased the bioaccessibility of the total antioxidants in FD and AD apples.

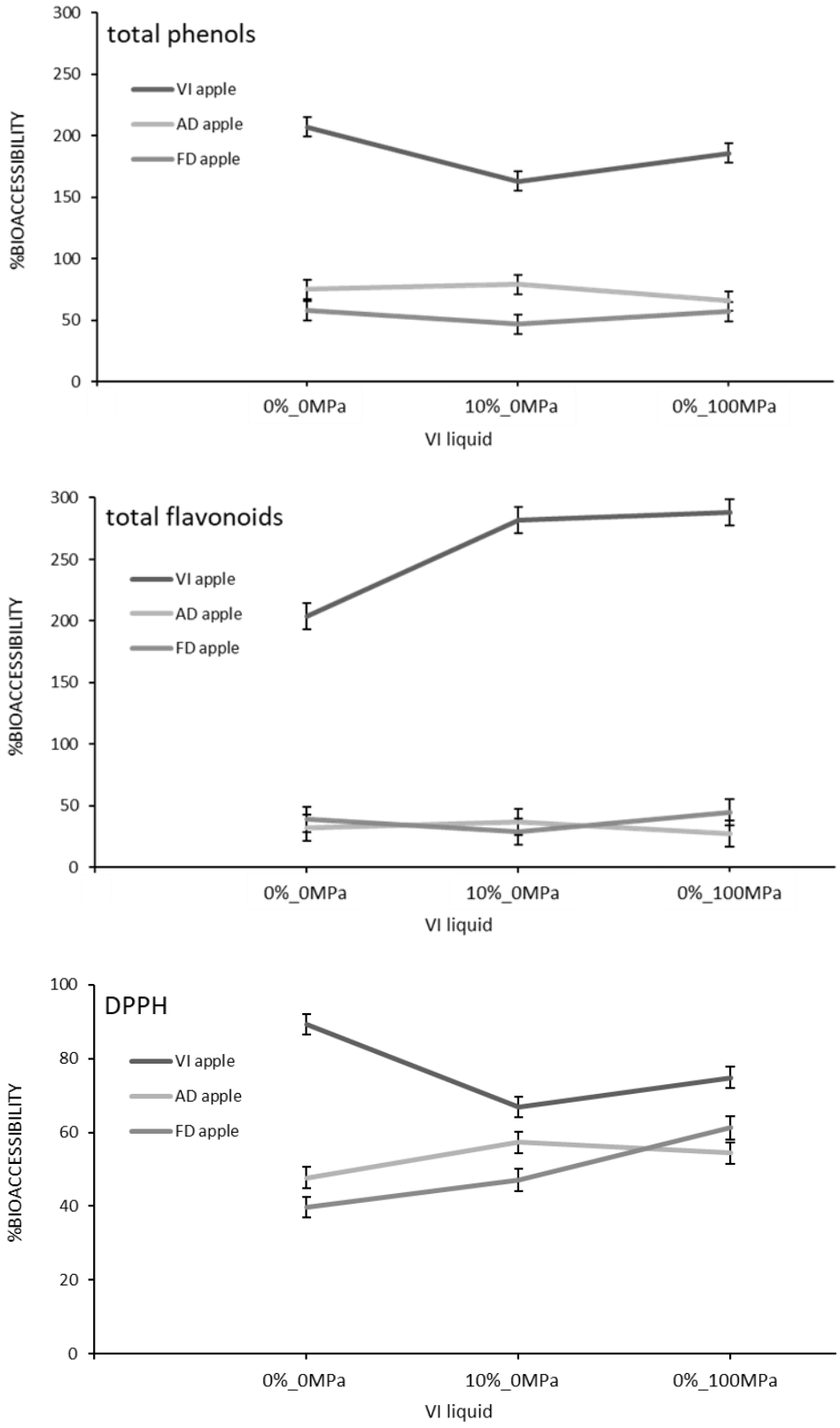


Figure 3. The bioaccessibility of antioxidants as affected by the food matrix (VI, AD, and FD apples) and the vacuum impregnation liquid (0%_0MPa, 10%_0MPa, and 0%_100MPa). Mean values and LSD intervals with a 95% confident level.

4. Conclusions

The study shows that both the survival of *L. salivarius* and the bioaccessibility antioxidants during *in vitro* digestion were affected by the food matrix of which they are a part. The greatest viability was found when the microorganism was incorporated into the apple's porous structure by means of the vacuum impregnation technique, but it decreased significantly with the application of a dehydration step. The bioaccessibility of antioxidants also reached the highest values in vacuum impregnated samples, in which both the total phenol and total flavonoid release to the intestinal liquid phase doubled that which was present in the undigested food. The addition of trehalose and the homogenization at 100 MPa increased the bioaccessibility of the total antioxidants in FD and AD apples and the bioaccessibility of the total flavonoids in the VI samples. Homogenizing at 100 MPa also increased the survival of *L. salivarius* during *in vitro* digestion in the FD samples.

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V. CONCLUSIONES

CONCLUSIONES GENERALES

- En conclusión, se puede afirmar que la fermentación del zumo de clementina comercial con *Lactobacillus salivarius* CECT 4063 durante 24 h es una herramienta útil para mejorar sus propiedades antioxidantes, al mismo tiempo que le confiere un potencial efecto probiótico.

- En general, ni el estrés osmótico debido a la presencia de trehalosa en el medio de crecimiento ni el estrés debido al gradiente de presión que supone la aplicación de altas presiones de homogeneización, mejoraron notablemente la concentración microbiana en el zumo, la adhesión del lactobacilo al epitelio intestinal o su capacidad para inhibir el crecimiento de *Helicobacter pylori.* Sin embargo, cada una de estas técnicas por separado sí logró aumentar la supervivencia de *Lactobacillus salivarius* CECT 4063 al proceso de digestión simulada *in vitro* y al almacenamiento en condiciones de refrigeración.

- Se puede añadir que la impregnación a vacío con zumo de clementina fermentado con *Lactobacillus salivarius* spp. *salivarius* CECT 4063 es una técnica adecuada para incorporar microorganismos con potencial efecto probiótico a la matriz estructural de láminas de manzana en cantidad suficiente como para poder ejercer un efecto beneficioso sobre la salud del consumidor.

- De entre las diferentes técnicas de deshidratación aplicadas para aumentar la estabilidad del producto impregnado, la liofilización sería la más recomendada pues permite mantener los recuentos por encima de 10^7 UFC/g al final del almacenamiento y tras la digestión *in vitro.*

- Como una alternativa de procesado más económica también se podría aplicar un secado con aire a 40 °C, lo que resultaría en un producto con unas propiedades organolépticas totalmente diferentes. En ese caso, lo recomendable sería añadir un 10% en peso de trehalosa al zumo de clementina antes de su inoculación y prolongar el secado hasta valores de actividad del agua en torno a 0,35 para lograr una concentración microbiana aceptable y que se mantenga estable durante el almacenamiento.

- Aunque la actividad antioxidante de las láminas de manzana impregnadas mejora significativamente tras la liofilización o el secado con aire a 40 °C, la baja estabilidad mostrada por los compuestos responsables de la misma durante el almacenamiento, unida a su escasa bioaccesibilidad, confirman que la ingesta del producto impregnado sea, a pesar de su elevada perecibilidad, es la opción más saludable.

GENERAL CONCLUSIONS

- In conclusion, it can be stated that the fermentation of commercial clementine juice with *Lactobacillus salivarius* CECT 4063 for 24 h is a useful tool to improve its antioxidant properties, while at the same time giving it a potential probiotic effect.
- In general, neither the osmotic stress due to the presence of trehalose in the growth medium nor the stress due to the pressure gradient caused by the application of high homogenization pressures markedly improved the microbial concentration in the juice, the adhesion of the lactobacillus to the intestinal epithelium or its ability to inhibit the growth of *Helicobacter pylori.* However, each of these techniques separately did manage to increase the survival of *Lactobacillus salivarius* CECT 4063 to the *in vitro* simulated digestion process and to storage under refrigeration conditions.
- It can be added that vacuum impregnation with clementine juice fermented with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 is a suitable technique for incorporating microorganisms with a potential probiotic effect into the structural matrix of apple slices in sufficient quantity to exert a beneficial effect on consumer health.
- Among the different dehydration techniques applied to increase the stability of the impregnated product, lyophilization would be the most recommended as it allows counts to be maintained above 10^7 CFU/g at the end of storage and after *in vitro* digestion.
- As a cheaper and alternative processing, air drying at 40 °C could also be applied, which would result in a product with completely different organoleptic properties. In that case, it would be advisable to add 10% by weight of trehalose to the clementine juice before its inoculation and prolong the drying until water activity values are around 0.35 to achieve an acceptable microbial concentration that remains stable during storage.
- Although the antioxidant activity of the impregnated apple slices improves significantly after freeze-drying or air-drying at 40 °C, the low stability shown by the compounds responsible for it during storage, together with their poor bioaccessibility, confirm that the intake of the impregnated product, despite its high perishability, is the healthiest option.