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Universidad del País Vasco

Euskal Herriko Unibertsitatea

Development of a microencapsulated functional ingredient formulated with the neurotransmitter GABA and the *Lactiplantibacillus plantarum K16* produced through biotechnological processes

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ABBREVIATIONS

AcP	Acetyl phosphate
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
API	Analytical Profile Index
Caco2	Human colon adenocarcinoma
CFU	Colony-forming units
DMEM	Dulbecco´s modified Eagle´s medium
EMP	Embden-Meyerhof-Parnas
EFSA	European Food Safety Authority
F_0-F1ATPase	F_0-F_1-adenosin triphosfatase
GRAS	Generally Regarded as Safe
GAD	Glutamic acid decarboxylase
GAP	Glyceraldehyde 3-phosphate
LAB	Lactic acid bacteria
L-DOPA	3,4-dihydroxy L-phenylalanine
L-Glu	L-glutamic acid
MSG	Monosodium glutamate
MRS	Man Rogosa Sharpe
MS	Mass spectrometry
OFAT	One-factor-at-a-time
SCFA	Short chain fatty acids

FAO/WHO	The Food and Agriculture Organization of the United Nations and the World Health Organization
ISAPP	The International Scientific Association for Probiotics and Prebiotics
UHPLC	Ultra-high performance liquid chromatography

RESUMEN

La salud humana se encuentra ampliamente relacionada con los microorganismos beneficiosos que habitan en el intestino, definiéndose como microbiota intestinal. Esta microbiota intestinal juega un papel esencial en la modulación del sistema inmunitario, diferentes rutas metabólicas y ayudando a prevenir a colonización de micoorganismos patógenos. Además, estos microorganismos simbióticos presentan una estrecha relación con diferentes órganos vitales. Es importante destacar la relación que existe entre el intestino y el cerebro, conocida como eje intestino-cerebro, creando una interconexión entre el sistema nervioso central y la comunidad microbiana de este entorno, ayudando a preservar la homeostasis en el sistema gastrointestinal. El desbalance de la microbiota intestinal desencadena la disrupción de la homeostasis, conocida como disbiosis, lo que favorece el desarrollo de enfermedades intestinales o extra-intestinal.

Los alimentos fermentados han sido utilizados ampliamente para prevenir y tratar enfermedades debido a la amplia comunidad microbiana junto con la variedad de compuestos bioactivos producidos por estos microorganismos que interactúan y favorecen la microbiota intestinal. Principalmente, los microorganismos que se encuentran en alimentos fermentados se clasifican como probióticos, que se pueden definir más concretamente como " microorganismos vivos que cuando son administrados en una concentración adecuada son capaces de producir un efecto beneficioso en el hospedador. Dentro de las especies probióticas más utilizadas cabe destacar las bifidobacteria y las bacterias ácido lácticas como son los *Lactobacillus, Lactococcus,* o *Streptococcus.* Estos probióticos pueden encontrarse en diferentes productos alimentarios o farmaceúticos, ya que se encuentran clasificados como microorganismos considerados seguros (GRAS) y además han sido clasificados con la presunción de seguridad. Dentro de la variedad de probióticos, *Lactiplantibacillus plantarum* se considera una especie importante debido a la amplia variedad de efectos beneficiososo para la salud humana observados en estudios *in vitro* e *in vivo.* Actualmente, los compuestos bioactivos sintetizados por probióticos son clasificados como postbióticos debido a su capacidad de promover efectos beneficiosos. Dentro de los postbióticos más prometedores cabe destacar los ácidos grasos de cadena corta, poliaminas, vitaminas, enzimas, bacteriocinas, neurotransmisores o amino ácidos.

El ácido gamma-aminobutírico (GABA) es un postbiótico destacable que puede ser producido por especies de *Lactobacillus* y *Bifidobacterium* a modo de protector en condiciones de estrés osmótico, en medio ácido o por falta de nutrientes. Por otro lado, el GABA desempeña un papel completamente diferente en los humanos, ya que se considera el neurotransmisor con mayor capacidad inhibidora en el sistema nervioso central. Este neurotransmisor se encarga de modular el comportamiento como puede ser el sueño, la memoria o el estado de ánimo, además de ayudar a prevenir el desarrollo de enfermedades del sistema cardiovascular, nervioso o endocrino. Estos efectos beneficiosos producidos por el GABA han atraído la atención de la industria alimentaria y farmaceútica, la cual se ha centrado en el desarrollo de nuevos suplementos enriquecidos con GABA. En un principio, la producción industrial de este neurotransmisor se llevaba a cabo mediante síntesis química. Sin embargo, el alto precio, el bajo rendimento y el impacto ambiental del proceso de producción ha conducido a la búsqueda de mejores alternativas. Por lo tanto, la producción de GABA se ha centrado en la síntesis biológica, usando principalmente microorganismos como las bacterias ácido lácticas que presentan una alta eficiencia de producción, un precio más reducido, bajo impacto ambiental, y además de ser seguros con clasificación GRAS. La producción de GABA por bacterias ácido lácticas se basa en un proceso biosintético donde una molécula de ácido glutámico es transportada al interior de la bacteria utilizando un antiportador. Dentro de la bacteria, la molécula de ácido glutámico se transforma en una molécula de GABA usando el enzima glutamato descarboxilasa, consumiendo un protón y liberando una molécula de dióxido de carbono. El rendimiento del proceso se encuentra ampliamente condicionado por parámetros de fermentación como es la temperatura de incubación, aditivos o tiempo de fermentación, los cuales necesitan ser optimizados para cada cepa utilizada, ya que son condiciones altamente cepa-dependientes.

Los efectos beneficiosos de los probióticos y postbióticos han desencadenado la apertura de un mercado global centrado en el desarrollo de productos alimenticios y farmaceúticos centrados en favorecer la salud humana. Por lo tanto, el primer paso en el proceso biotecnológico es identificar y aislar el probiótico y postbiótico más adecuados para desempeñar un efecto específico en la salud. Luego, se debe de seleccionar el medio de cultivo más idóneo para favorecer la máxima producción de biomasa. Generalmente, las bacterias ácido lácticas como *L. plantarum* necesitan un medio con una alta concentración de nutrientes como es el caso del medio Man Rogosa Sharpe (MRS), cuyo

uso aumenta considerablemente los costes del proceso de producción a escala industrial. Por tanto, una de las alternativas más atractivas para sustituir el medio MRS es la reutilización de subproductos agroalimentarios como sustratos de fermentación. La revalorización de estos subproductos como medios de cultivo supone una forma de dar valor añadido a productos de bajo coste que presentan una amplia variedad de nutrientes, lo cual, además, favorece la disminución de residuos y reduce el impacto ambiental generado por la destrucción de los mismos.

Una vez realizado el proceso de fermentación, la biomasa microbiana y los compuestos bioactivos producidos por estos microorganismos necesitan ser recuperados y almacenados manteniendo su integridad. Por otro lado, es necesario realizar estudios de resistencia de los probióticos y postbióticos frente a condiciones adversas como son las presentes en el sistema gastrointestinal, ya que ambos necesitan llegar con funcionalidad suficiente al intestino donde realizarán su efecto beneficioso. Para mantener la viabilidad de los probióticos y la estabilidad de los postbióticos se puede desarrollar microcápsulas protectoras. Estas microcápsulas pueden ser producidas utilizando diferentes métodos, materiales biopoliméricos, o características dependiendo de los requerimientos del producto final. El proceso biotecnológico da lugar a un ingrediente funcional que podrá formar parte de alimentos funcionales, fármacos o suplementos alimentarios.

De acuerdo con las tendencias actuales, esta tesis doctoral se ha centrado en el desarrollo de un nuevo ingrediente funcional microencapsulado enriquecido con una cepa de *L. plantarum (K16)* y el postbiótico GABA. Para ello, se ha llevado a cabo un proceso biotecnológico que ha englobado diferentes objetivos, desde la identificación y aislamiento de bacterias ácido lácticas productoras de GABA, caracterización de la actividad probiótica de la cepa aislada, estudio de la producción de GABA para la identificación de los parámetros más influyentes usando diferentes medios de fermentación, hasta el diseño de un nuevo ingrediente microencapsulado compuesto de la cepa bacteriana seleccionada y de GABA producido.

Para alcanzar estos objetivos en primer lugar se llevó a cabo una búsqueda bibliográfica extensiva centrada en encontrar los microorganismos más interesantes para ser aislados, como cepas de *Lactobacillus spp*, y los beneficios más destacables del postbiótico GABA. A partir de esta búsqueda, se llevó a cabo la preparación de kimchi (alimento fermentado) con materia natural autóctona y se utilizó para aislar bacterias ácido lácticas. El aislamiento y la selección de bacterias ácido lácticas se llevó cabo en

primer lugar por la identificación de la producción de ácido láctico, evaluación de la presencia de catalasa y mediante la tinción Gram. Una vez seleccionadas bacterias productoras de ácido láctico, catalasa negativas y Gram positivas, se realizó una prueba de producción de GABA usando medio MRS. En este estudio, solo una cepa presentó la capacidad de producir GABA y fue secuenciada e identificada como *Lactiplantibacillus plantarum K16.* A partir de aquí, se realizó un estudio de caracterización de *L. plantarum K16* para determinar si era segura para su uso y si presentaba potencial efecto beneficioso, y podía ser clasificada como probiótico.

Los estudios de caracterización de *L. plantarum K16* indicaron que esta bacteria podía ser considerada inocua debido a su falta de actividad hemolítica y las resistencias a antibióticos que presentaba. Por un lado, estas resistencias podrían considerarse beneficiosas en el caso de utilizar a *L. plantarum K16* para favorecer el mantenimiento de la microbiota intestinal junto con un tratamiento de antibióticos. Sin embargo, previamente sería necesario realizar un estudio de la concentración mínima inhibitoria de cada uno de los antibióticos estudiados y determinar la posibilidad de transmitir las resistencias de antibióticos al huésped.

L. plantarum K16 también presentó un importante potencial a la hora de metabolizar diferentes carbohidratos, llegando a consumir monosacáridos como son la glucosa, galactosa o fructosa, hasta moléculas más complejas como oligosacáridos como la rafinosa, polisacáridos como la inulina o glucósidos como la amigdalina. El consumo de monosacáridos favorece a la obtención rápida de energía favoreciendo el crecimiento del microorganismo. Sin embargo, el consumo de moléculas más complejas puede favorecer a la absorción de nutrientes difíciles de asimilar por su complejidad, estimular el proceso de digestión, favorecer el mantenimiento de la microbiota intestinal y favorecer la producción de ácidos orgánicos beneficiosos. Además, otros enzimas presentes en *L. plantarum K16* también pueden desempeñar un efecto beneficioso. Por ejemplo, se ha observado que esta bacteria presentaba ciertas enzimas que pueden favorecer el metabolismo de lipoproteínas, reducir la intolerancia a la lactosa reduciendo la lactosa y evitar la colonización de micoorganismos patógenos atacando a la pared celular. Además, en los estudios antimicrobianos *in vitro* realizados frente a diferentes microorganismos patógenos comunes, se observó la capacidad inhibitoria de *L. plantarum K16,* especialmente frente a *Salmonella typymurihum.*

Paralelamente, se realizó la evaluación de la producción de GABA por *L. plantarum K16* usando técnicas de fermentación. En primer lugar, se realizó un diseño experimental centrado en el estudio individual de siete factores (temperatura de incubación, concentración de extracto de levadura, tiempo de fermentación, porcentaje de inóculo, pH inicial, concentración de glutamato monosódico y glucosa) usando como medio de cultivo MRS comercial. Es diseño experimental también fue útil para identificar las condiciones óptimas de cada parámetro y así maximizar la producción de GABA por *L. plantarum K16,* llegando a alcanzar aproximadamente 2115 mg/L de GABA.

Una vez realizado el estudio con medio MRS comercial, se realizó una prueba de producción utilizando subproductos agroalimentarios (tomate, manzana, naranja y pimiento verde) como sustratos de fermentación para la obtención de GABA. Dentro de los subproductos utilizados, el subproducto de tomate presentó una mayor producción de GABA. De acuerdo con estos resultados, el subproducto de tomate fue seleccionado como sustrato de fermentación para el desarrollo del ingrediente funcional. A continuación, se realizó un estudio del crecimiento de *L. plantarum K16* y su producción de GABA usando medios de cultivo con subproducto de tomate. Atendiendo al crecimiento de la bacteria en subproducto de tomate, se llevó a cabo un estudio de interconexión entre diferentes concentraciones de glucosa, extracto de levadura y minerales, y el crecimiento microbiano. Los resultados de este estudio indicaron que el crecimiento de *L. plantarum K16,* usando medios de cultivo con subproducto de tomate, se encontraba significativamente relacionado con la concentración presente de minerales y, en un segundo lugar, por la cantidad de glucosa añadida. En el caso de la producción de GABA, el estudio de interconexión se realizó entre diferentes concentraciones de glucosa, extracto de levadura y glutamato monosódico. Este experimentó indicó que la síntesis de GABA por *L. plantarum K16* se encuentra positivamente relacionada con la concentración de extracto de levadura y glutamato monosódico. Sin embargo, una mayor concentración de glucosa presentaba una actividad inhibitoria de la producción de GABA. Con estos estudios se identificaron las mejores condiciones para la obtención del mayor crecimiento microbiano y rendimiento de síntesis de GABA, llegando a alcanzar hasta 9,5 log unidades formadoras de colonias por mililitro y 1776 mg/L de GABA, respectivamente.

Finalmente, para realizar el desarrollo de un ingrediente funcional, es necesario asegurarse que los probióticos y los compuestos bioactivos suplementados, son capaces

de soportar las condiciones intestinales adversas. Para ello, se hicieron estudios de simulación intestinal *in vitro* con *L. plantarum K16* y GABA, y así observar su resistencia a estas condiciones adversas. En este caso, los resultados mostraron que *L. plantarum K16* tenía estabilidad frente a las condiciones gástricas menos ácidas (pH 4 y pH 6) y las condiciones intestinales. Aunque en condiciones gástricas con pH 2, se observó que al cabo de 120 min se producía la disminución de la viabilidad de *L. plantarum K16.* De igual forma, se evaluó la estabilidad de GABA bajo las mismas condiciones, el cual mostró una amplia inestabilidad frente a todas las condiciones gastrointestinales estudiadas.

De acuerdo con estos estudios, se llevó a cabo el diseño y la producción de una microcápsula compuesta de biomas de *L. plantarum K16* recuperada de su crecimiento en subproducto de tomate bajo condiciones previamente optimizadas. Esta cápsula también contenía subproducto de tomate enriquecido con GABA previamente producido usando las condiciones previamente optimizadas. Una vez mezcladas la biomasa de *L. plantarum K16* y el subproducto de tomate enriquecido con GABA, se añadió a la mezcla un biopolímero (alginato) para llevar a cabo la producción de las cápsulas mediante técnicas de extrusión por vibración de boquilla. Por tanto, la mezcla a encapsular saldría a presión por una boquilla que estaría sometida a una frecuencia, generando la vibración de la misma y creando gotas. La caída de estas gotas en un baño de endurecimiento dio lugar a la creación de microcápsulas, que fueron recuperadas y sometidas a un baño de leche para proteger los microorganismos en el proceso de secado. Finalmente, las cápsulas fueron liofilizadas, dando lugar a un ingrediente funcional apto para ser utilizado en alimentos funcionales, fármacos y diferentes suplementos alimentarios.

Con los resultados obtenidos en esta tesis doctoral se puede concluir que el alimento fermentado kimchi es una buena fuente de bacterias ácido lácticas, destacando la bacteria identificada como *Lactiplantibacillus plantarum K16* que presentaba la capacidad de producir GABA. Además, los estudios de seguridad y capacidad probiótica de esta cepa indicaban que se trata de una cepa inocua y potencialmente útil para promover la salud humana. Por otro lado, se identificaron las condiciones óptimas de producción de GABA en MRS y, lo que es más importante, se observó como subproductos agroalimentarios, más específicamente el tomate, son buenos sustratos de fermentación para la producción de postbióticos como el GABA. Finalmente, se pudo

desarrollar una microcápsula protectora para asegurar que GABA y *L. plantarum K16*, son capaces de llegar funcionales al intestino y realizar su efecto beneficioso.

SUMMARY

Human health has been directly related to beneficial microorganisms that reside in the intestine, defined as gut microbiota . This gut microbiota plays a vital role in modulating the immune system, stimulating different metabolic pathways or preventing pathogens' colonisation. Likewise, several vital organs are strongly linked to these symbiotic microorganisms, highlighting the close relationship between the brain and the gut microbiota, known as the gut microbiota-brain axis, creating an interconnection between the central nervous system and the microbial community to preserve the homeostasis in the gastrointestinal tract. The imbalance of the gut microbiota triggers the disruption of homeostasis, defined as dysbiosis, which enhances the development of intestinal or extra-intestinal disorders.

Fermented foods have been widely used to prevent and treat illnesses due to their great microbial community and the bioactive metabolites produced by these microorganisms, which interact with the gut microbiota. Mainly, the beneficial microorganisms found in fermented foods are classified as probiotics, defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Bifidobacteria and lactic acid bacteria (LAB), such as *Lactobacillus, Lactococcus or Streptococcus,* are the most used. These probiotic microorganisms are widely found in food and pharmaceutical products because they are generally considered safe microorganisms (GRAS) and have been classified as a qualified presumption of safety. Among probiotics, *Lactiplantibacillus plantarum* is an important specie because it presents various beneficial effects observed *in vitro* and *in vivo* studies. Moreover, bioactive metabolites, defined as postbiotics, produced by probiotics, such as *L. plantarum,* are gaining interest due to their promoting health effect. Short-chain fatty acids, polyamines, vitamins, enzymes, bacteriocins, neurotransmitters or amino acids are some of the most promising postbiotic metabolites.

The gamma-aminobutyric acid (GABA) is an interesting postbiotic metabolite synthesised by *Lactobacillus* and *Bifidobacterium* species as a protective mechanism against stressful situations such as acid, osmotic or starvation. Furthermore, GABA plays a different role in human health as it is considered the most crucial inhibitory neurotransmitter in the central nervous system. This neurotransmitter modulates behaviour such as mood, sleep and memory and prevents the development of

cardiovascular, nervous or endocrinological systems. The benefits conferred by GABA have caught the attention of the food and pharmaceutical industry, which has focused on developing new supplements enriched with GABA. In the beginning, the industrial production of this neurotransmitter was performed through chemical synthesis.

Nevertheless, the high price, the low yield and the great environmental impact of the process lead to looking for better alternatives. Hence, GABA production was moved to biological synthesis, mainly using microorganisms, such as LAB, due to their excellent efficiency, affordable cost, low environmental impact, and GRAS classification. The GABA production of LAB is a biosynthetic pathway where a molecule of glutamic acid (L-Glu) is transported through an antiporter into the microorganisms. Inside the bacteria, the molecule of L-Glu is transformed into GABA using a glutamic acid decarboxylase enzyme, consuming at the same time a proton and releasing a carbon dioxide molecule. The yield of this biosynthetic process is closely related to fermentation parameters, such as incubation temperature, additives or fermentation time, which need to be optimised as these conditions are strain-dependent.

The beneficial effect of probiotics and postbiotics has led to the opening of a widespread market where food and pharmaceutical products are developed to enhance human health. Therefore, the first step in the biotechnological process is to isolate and select the most suitable probiotic and postbiotic metabolite to perform a specific health effect. Then, the fermentation media is chosen to ensure the production of the highest biomass concentration. Generally, LAB, *L. plantarum*, are grown using a high-nutrient media known as Man Rogosa Sharpe (MRS), increasing the production cost while the fermentation is scaled up. Therefore, one of the most attractive alternatives is reusing agri-food by-products as fermentation substrates, which is an excellent way to revalue inexpensive products with great nutrient composition and reduce the environmental impact produced by destroying these by-products.

Afterwards, the microbial biomass and the bioactive metabolites must be recovered and stored. Then, studies need to be conducted to ensure the resistance of the probiotic and the postbiotic against the gastrointestinal tract to arrive functional to the gut, which will perform a beneficial effect. Furthermore, the viability of probiotics and the stability of postbiotics could be preserved by designing a protective capsule. Depending on the final product's requirements, these capsules could be produced using different techniques, materials or characteristics. The end product of this biotechnological

process results in a functional ingredient which can be used as part of functional food, drugs or dietary supplements.

Following current trends, this Ph.D. thesis focused on developing a new microencapsulated functional ingredient enriched with an *L. plantarum* (K16) strain and the postbiotic metabolite GABA. For this purpose, a biotechnological process was carried out, encompassed different objectives, from the identification and isolation of GABA-producing lactic acid bacteria, characterisation of the probiotic activity of the isolated strain, study of the GABA production for the identification of the most influential parameters using different fermentation media, to final design of a new microencapsulated ingredient composed of the selected bacterial strain and the GABA produced.

An extensive bibliographic search was first carried out to achieve these objectives, focused on finding the most interesting microorganisms to be isolated, such as *Lactobacillus spp* strains, and the most notable benefits of postbiotic GABA. First, the preparation of kimchi (a fermented food) with raw natural material was performed to isolate lactic acid bacteria. Next, the isolation and selection of lactic acid bacteria were conducted by identifying the production of lactic acid, evaluating the presence of catalase and by Gram staining. Once Gram-positive and catalase-negative lactic acid-producing bacteria were selected, a GABA production test was performed using MRS broth. In this study, only one strain could produce GABA and was sequenced and identified as *Lactiplantibacillus plantarum K16*. From here, a characterisation study of *L. plantarum K16* was carried out to determine its safety and if it had a potential beneficial effect and could be classified as a probiotic.

The characterisation studies of *L. plantarum K16* indicated that this bacterium could be considered innocuous due to its lack of haemolytic activity and some antibiotic resistance. On the one hand, these resistances could be regarded as beneficial in the case of using *L. plantarum K16* to favour the maintenance of the intestinal microbiota with antibiotic treatment. However, on the other hand, it would previously be necessary to study the minimum inhibitory concentration of each antibiotic studied and determine the possibility of transmitting antibiotic resistance to the host.

L. plantarum K16 also showed potential promoting health effects by metabolising different carbohydrates like monosaccharides such as glucose, galactose or fructose. Even

more complex molecules, such as oligosaccharides (raffinose), polysaccharides (inulin) or glycosides (amygdalin), were consumed by these microorganisms. The consumption of monosaccharides could enhance the fast obtention of energy, favouring the growth of the microorganism. However, the consumption of more complex molecules can increase the absorption of nutrients that are difficult to assimilate due to their complexity, stimulate the digestion process, maintain the intestinal microbiota, and produce beneficial organic acids. Furthermore, other enzymes in *L. plantarum K16* may also have a beneficial effect. For example, it has been observed that this bacterium has certain enzymes that can promote lipoprotein metabolism, reduce lactose intolerance by reducing lactose, and prevent the colonisation of pathogenic microorganisms by attacking the cell wall. In addition, *in vitro* antimicrobial studies against different common pathogenic microorganisms showed the inhibitory capacity of *L. plantarum K16*, especially against *Salmonella typymurihum*.

In parallel, the evaluation of GABA production by *L. plantarum K16* was conducted using fermentation techniques. First, an experimental design focused on the individual study of seven factors (incubation temperature, yeast extract concentration, fermentation time, percentage of inoculum, initial pH, and concentration of monosodium glutamate and glucose) was done using commercial MRS broth. The experimental design was also useful in identifying the optimal conditions for each parameter and thus maximise GABA production by *L. plantarum K16*, reaching approximately 2115 mg/L of GABA.

Once the study was performed with a commercial MRS medium, a production test was carried out using agri-food by-products (tomato, apple, orange and green pepper) as fermentation substrates to obtain GABA. Among the by-products used, the tomato by-product presented a higher production of GABA. According to these results, the tomato by-product was selected as the fermentation substrate for developing the final functional ingredient. Next, a study of *L. plantarum K16* growth and its production of GABA was done using tomato by-product as a fermentation substrate. Therefore, an interconnection study between different concentrations of glucose, yeast extract and minerals was performed to evaluate their effect in microbial cell growth using tomato by-product. The results of this study indicated that the growth of *L. plantarum K16*, using tomato by-product as the fermentation substrate, was significantly related to the concentration of minerals present and, secondarily, to the amount of added glucose. In the case of GABA

production, the interconnection study was performed between different concentrations of glucose, yeast extract, and monosodium glutamate. This experiment indicated that GABA synthesis by *L. plantarum K16* was positively related to yeast extract concentration and monosodium glutamate. However, a higher glucose concentration exhibited inhibitory activity on GABA production. These studies identified the best conditions for obtaining the highest microbial growth and GABA yield, reaching up to 9.5 log colony-forming units *per* millilitre and 1776 mg/L of GABA, respectively.

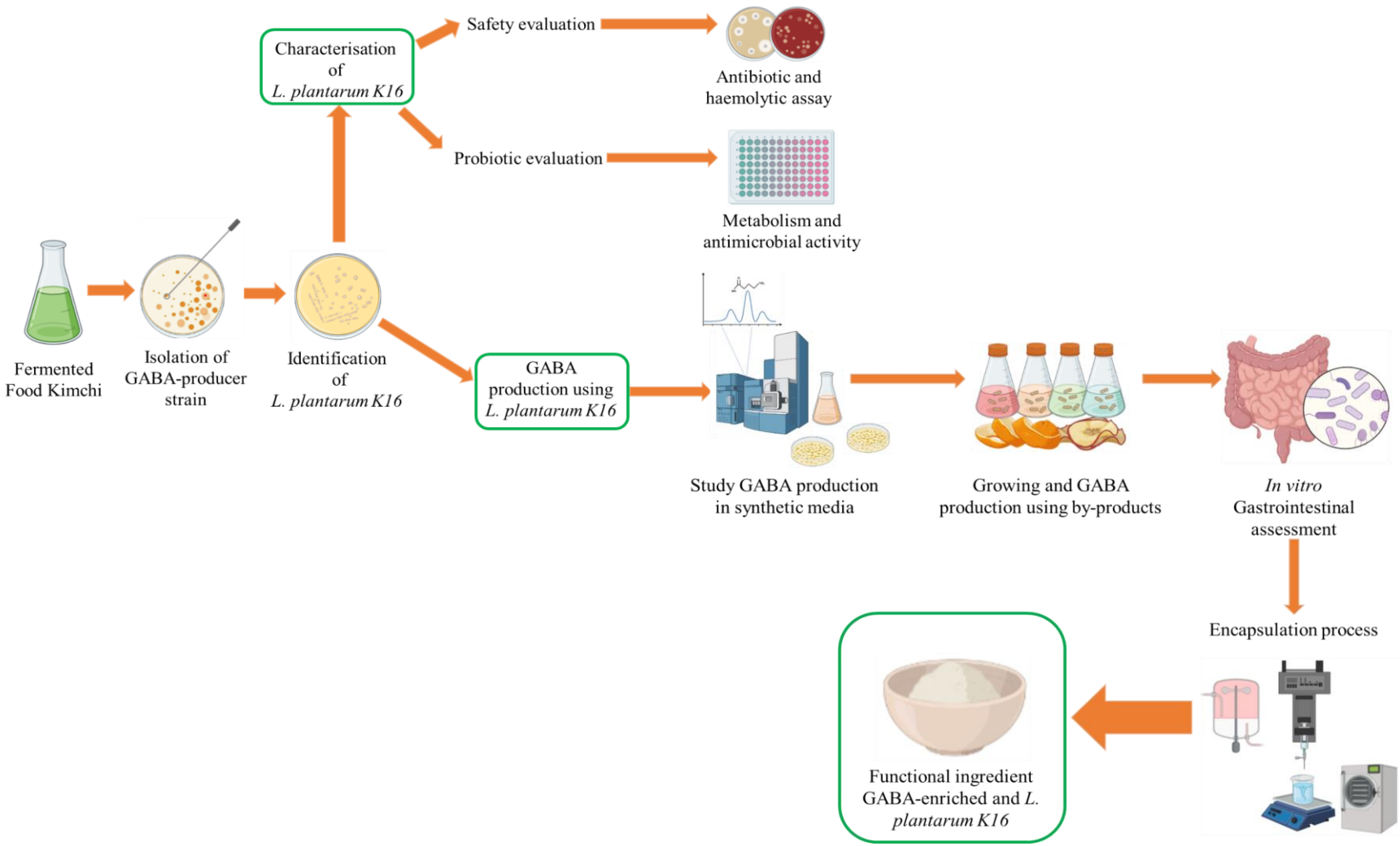
Finally, to develop a functional ingredient, it is necessary to ensure that the supplemented probiotics and bioactive compounds can withstand adverse intestinal conditions. To this end, *in vitro* intestinal simulation studies were carried out with *L. plantarum K16* and GABA; thus, their resistance to these adverse conditions was observed. In this case, the results showed that *L. plantarum K16* had stability against less acid gastric conditions (pH 4 and pH 6) and intestinal conditions. Although under gastric conditions with pH 2, it was observed that after 120 min, the viability of *L. plantarum K16* decreased. Similarly, the stability of GABA was evaluated under the same conditions, which showed a wide instability against all the gastrointestinal conditions studied.

According to these studies, designing and producing a microcapsule composed of *L. plantarum K16* biomass recovered from its growth in tomato by-product under previously optimised conditions was necessary. This capsule also contained GABA-enriched tomato by-product previously produced using the optimised conditions. Once the biomass of *L. plantarum K16* and the GABA-enriched tomato by-product were mixed, a biopolymer (alginate) was added to the mixture to carry out the production of the capsules using extrusion techniques by vibration technologies. Therefore, the encapsulation mixture would come out under pressure through a nozzle subjected to a frequency, generating its vibration and creating drops. The fall of these drops in a hardening bath led to the creation of microcapsules, which were recovered and subjected to a milk bath to protect the microorganisms in the drying process. Finally, the capsules were freeze-dried, giving rise to a functional ingredient suitable for functional foods, drugs and food supplements.

With the results obtained in this Ph.D. thesis, it can be concluded that kimchi fermented food is a good source of lactic acid bacteria, highlighting the bacteria identified as *Lactiplantibacillus plantarum K16*, which could produce GABA. In addition, studies

on this strain's safety and probiotic capacity indicated that it is a harmless and potentially useful strain for promoting human health. On the other hand, the optimal conditions for GABA production in MRS broth were identified. More importantly, it was observed that agri-food by-products, specific tomato, were good fermentation substrates for producing postbiotics such as GABA. Finally, it was possible to develop a protective microcapsule to ensure that GABA and L. *plantarum K16* were able to reach the intestine functionally and perform their beneficial effect.

GRAPHICAL ABSTRACT



SECTION I

1. STATE OF THE ART

1.1. Importance of intestinal microbiota for human health

Human health is broadly linked to the balance of the symbiotic microorganisms (archaea, fungi, bacteria and viruses) that reside in the intestine, known as the gut microbiota (Morais et al., 2021). One of the essential functions of gut microbiota is the modulation of the immune system, metabolism promotion, protection against the colonization of pathogens, and enhancing the correct functioning of other organs such as the liver, bone, or lungs (Gensollen et al., 2016). Furthermore, the gut microbiota has significant crosstalk with the brain, known as the gut microbiota-brain axis , that enhances the preservation of the homeostasis of the gastrointestinal tract, central nervous systems and the microbial community (Chávarri et al., 2021; Philip & Bercik, 2017). The gut microbiota-brain axis presents several communication routes (Figure 1) through the autonomous system, highlighting the relationship between the enteric nervous system and the vagus nerve, immune system, and neuroendocrine or hypothalamic-pituitary-adrenal axis (Carabottia et al., 2015). For instance, the vagus nerve plays a key role in the gut microbiota-brain axis by stimulating the releasement of metabolites, like short-chain fatty acids (SCFA), neurotransmitters, such as dopamine, serotonin or gamma-aminobutyric acid (GABA), or hormones (corticotropin releasement hormone) that directly affect the gut microbiota (Hyland & Cryan, 2010; Liu et al., 2019).

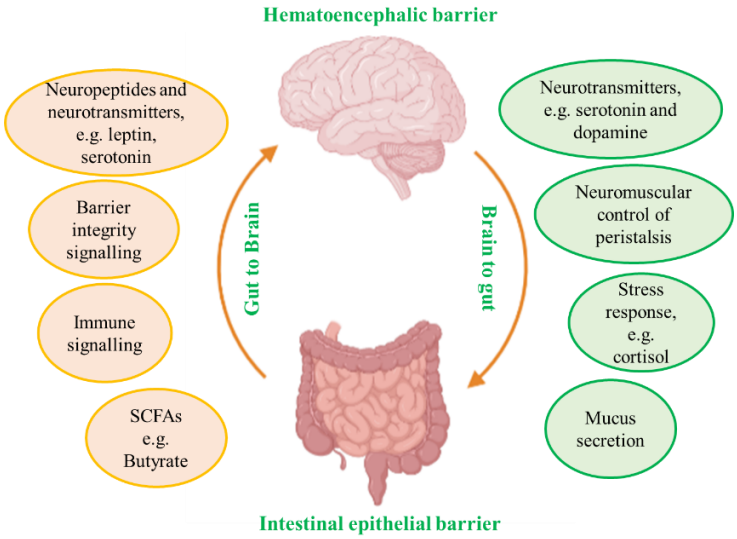


Figure 1: Gut microbiota-brain axis communication routes (image adapted from our book chapter Chávarri et al., 2021)

The homeostasis of the gut microbiota-brain axis could be affected by the disruption of the proper balance of the microbial community (Gagliardi et al., 2018). The imbalance of the gut microbiota has been defined as dysbiosis triggered by intestinal or extra-intestinal diseases, which threaten normal physiological functioning (Carding et al., 2015). Heinen et al. (2020) reported that the microbial community of fermented foods and the bioactive metabolites produced by these microorganisms could interact with the gut microbiota, maintaining an adequate microbial balance. In addition, the importance of these microorganisms and their beneficial metabolites has been highlighted in the review article (Annex I.I) in which the importance of the postbiotic neurotransmitter GABA and the probiotic *Lactiplantibacillus plantarum* are detailed.

1.2. Fermented foods and diversity of probiotics

Fermented foods have been widely consumed since the Hippocratic Corpus of Ancient Greece. A considerable variety of fermented foods has been observed worldwide, with more than 5,000 types related to traditions and cultural differences (Bell et al., 2017). For example, Korea, China, and Japan normally consume more plant-based fermented foods. However, Europe, North-Central America and the Middle East have developed more fermented dairy products (Rul et al., 2022). The acquired importance of fermented foods worldwide is due to their high diversity of potential beneficial health effects, such as the prevention and treatment of illnesses through the protection against oxidative stress, regulation of cellular metabolism, modulation of the immune system and cognitive support (Wilburn & Ryan, 2017). The International Scientific Association for Probiotics and Prebiotics (ISAPP) highlighted that these beneficial health effects are attributed to the microorganisms in fermented foods and the bioactive compounds they can release during fermentation. Hence, the food composition, microbial strain, or fermentation parameters could modulate the health-promoting effect (Marco et al., 2021). Table **1** shows some relevant fermented foods, the beneficial microorganisms found in them and their potential health-promoting effects.

Various beneficial microorganisms such as bacteria, yeasts and fungi are generally found in fermented foods, typically classified as probiotics (Chilton et al., 2015). The ISAPP (2014) supported the definition proposed by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) in 2002, where they claimed that probiotics are "live microorganisms which, when administered in adequate

amounts, confer a health benefit on the host" (FAO/WHO, 2002; Hill et al., 2014). Currently, bacteria, such as bifidobacteria and lactic acid bacteria (LAB), are the main microorganisms used as probiotics (Kosmerl et al., 2021).

Table 1: Common microorganisms found in fermented foods and their potential beneficial effects on preserving a healthy gut microbiota

Fermented food	***Microorganisms***	***Beneficial health effect***	***References***
Kimchi	Lactobacillus, Leuconostoc, Weisella, Pediococcus	Antioxidant activity, inhibition of pro-inflammatory cytokines, cholesterol reduction, liver injury attenuation	(Lee et al., 2020)
Kefir	Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, Candida, Kluveromyces, Saccharomyces	Antibacterial activity, immunomodulatory effect, relieved gastrointestinal disorders	(Guclu et al., 2021)
Kombucha	Bacillus, Acetobacter, Gluconobacter, Aspergillus	Antimicrobial effect, detox activity, enhance the gastrointestinal, cardiac, hepatic, and neurologic functions	(Kaashyap et al., 2021)
Miso	Bacillus, Lactobacillus, Leuconostoc, Enterococcus, Aspergillus, Zygosaccharomyces	Brain and kidney protection, stroke prevention, anti-diabetic	(Allwood et al., 2021)
Sourdough	Weisella, Lactobacillus, Lactococcus, Streptococcus, Leuconostoc	Metabolism regulation, gastrointestinal benefits, control glycaemic index	(Lau et al., 2021)

The *Bifidobacterium* genera (Actinobacteria Phylum) is a wide group of catalase-negative, non-spore-forming and gram-positive curved and bifurcated rod-shaped anaerobic bacteria which play a key role in the gut microbiota (Ventura et al., 2015). This genus is closely related to LAB, however, the metabolism of sugars by *Bifidobacterium* is more focused on the production of acetic acid than lactic acid (Hoover, 2014).

On the other hand, LABs (Firmicutes Phylum) are non-spore-forming, gram-positive and catalase-negative aerotolerant or microaerophilic bacteria which highly produce lactic acid from sugar fermentation. Bacilli or cocci are included in this group, being essential to highlight the genera *Oenococcus, Pediococcus, Weisella, Leuconostoc, Lactococcus, Streptococcus* or *Lactobacillus* (Ayivi et al., 2020). Also, microorganisms from the *Bacillus* genera (Firmicutes phylum), known as catalase-positive, spore-forming, and gram-positive, have attracted attention to their use as probiotics (Lu et al., 2018). Several yeast and fungi, such as *Saccharomyces cerevisiae, S. boulardi, Kluyveromyces lactis or Aspergillus oryzae*, also present probiotic effects. Nevertheless, *S. boulardi* is the only yeast properly classified as a human probiotic (Dawood et al., 2020; Homayouni-Rad et al., 2020; Sen & Mansell, 2020).

Furthermore, the microorganisms used as probiotics should be considered Generally Regarded as Safe (GRAS). The European Food Safety Authority (EFSA) has included *Lactobacillus, Bifidobacterium,* and *Bacillus* species in the Qualified Presumption of Safety status (Liu et al., 2020; Ruiz Sella et al., 2021). However, FAO/WHO, (2006) highlights that it is essential to perform an *in vitro* characterization before carrying out *in vivo* trials. For instance, it is necessary to determine the resistance against stressful situations, protection against pathogens or modulation of the immune system (James & Wang, 2019). Surve et al. (2022) performed a safety assessment of two *L. plantarum* strains isolated from Indian foods by evaluating their haemolytic activity, production of biogenic amines and resistance against antibiotics. Won et al. (2020) focused on the characterization of *L. sakei* on the resistance of this strain against different concentrations of bile salts and the variation of pH, as well as the production of enzymes with a potential health effect. On the other hand, Jamyuang et al. (2019) also evaluated the probiotic effect of *Lactobacillus* strains isolated from human breast milk. In this case, a model of epithelial cells was used to determine how *Lactobacillus* could adhere to this kind of cells and protect then against the colonization of enteric pathogens.

Moreover, Yang et al. (2021) performed an *in vivo* study with rats to confirm the antioxidant effect of *L. paracesei*, isolated from fermented rice, by reducing the expression of genes involved in oxidative stress. Chaudhari et al. (2022) used Swiss albino mice and Wistar rats to evaluate the antidiarrheal effect of *B. coagulans*. The results showed that *B. coagulans* could increase gut integrity by repairing damaged intestinal cells and improving the integrity of the colon goblet cells. Lee and Lee (2022)

analysed the probiotic effect of *S. cerevisiae* using a mice model that presented induced colitis. The supplementation of *S. cerevisiae* reduced the secretion of pro-inflammatory cytokines, improved the functionality of proteins essential for a healthy gut barrier and helped recover the structure of a normal colon. Chávarri et al. (2022) also have reported the importance of probiotics in the treatment and prevention of nutritional health disorders such as undernutrition (severe acute malnutrition in children, pregnancy and elderly), overnutrition (cardiovascular and metabolic disorders) or malnutrition associated with other disorders (pathogen infection, food intolerance, irritable bowel diseases). Within the great variety of probiotic microorganisms and their potential beneficial health effect, *L. plantarum* is of great interest due to the high versatility and relevant health effect of this species (Darby & Jones, 2017).

1.3. *Lactiplantibacillus plantarum*

In the beginning, *Lactiplantibacillus plantarum* was named *Streptobacterium plantarum*, and this name was changed in the 1980s to *Lactobacillus plantarum* because of the phenotypic similarities between other *Lactobacillus* species (Todorov & de Melo Franco, 2010). Recently, Zheng et al. (2020) conducted an in-depth phylogenetic analysis and finally changed the name to *Lactiplantibacillus plantarum.* Furthermore, *L. plantarum* inhabits a wide range of niches such as meat, dairy products, vegetables, and some parts of the human body. Also, they are mainly found in vegetable-based fermented foods, such as kimchi, sauerkraut, brined olives, sourdough, or stockfish (Khemariya et al., 2016). Likewise, *L. plantarum* strains highlight their great adaptability to a wide range of environments, may be because this specie has a larger genome size, which ranges between 2.91 to 3.70 Mb, compared to other LAB (Bringel et al., 2001).

1.3.1 Beneficial effects on human health

The *L. plantarum* specie is characterised due to its demonstrated probiotic effect such as protection against pathogenic colonisation (Zhao et al., 2022), adhesion to the gastrointestinal epithelium (Santarmaki et al., 2017), antioxidant effect (Luan et al., 2021), immunomodulatory activity (Villena et al., 2017), or reduction of the blood pressure (Zareian et al., 2015). For instance, Li et al. (2012) showed the antioxidant effect of *L. plantarum*, isolated from traditional Chinese fermented foods, in senescent mice. The administration of this bacteria, which was high resistance against hydrogen peroxide, reduced the oxidative stress by stimulating the superoxidase dismutase, the glutathione

peroxidase and the general antioxidant activity in the mice liver. Liu et al. (2016) tested the neuroprotective effect and the blood-pressure modulation of *L. plantarum TWK10* strain using hypertensive induced rats. After the administration of this strain, the production of nitric oxide in plasma was enhanced, coupled with the inhibition of the angiotensin-converting enzyme in serum and, thus, a significant reduction of the blood pressure. Hong et al. (2015) and Wang et al. (2021b) also observed the neuroprotective effect of *L. plantarum* strains, in murine models, by activating signaling pathways or enhancing the expression of regulation genes.

Plenty of clinical trials have also been performed to determine the beneficial effect of *L. plantarum* strains. Darby and Jones (2017) summarised successful clinical trials where this bacteria reduced inflammatory markers and decreased lipid levels in blood, protected against cardiovascular diseases, fought against severe infections and preserved the gastrointestinal tract. Sohn et al. (2022) showed that the administration of *L. plantarum K50* strain for 12 weeks to obese patients significantly reduced the levels of triglyceride and cholesterol coupled with an increase of *L. plantarum* and a reduction of *Actinobacteria* in the intestinal community. Liu et al. (2021) detected a reinforcement of the gut microbiota by increasing the concentration of butyric acid producers and alleviating the symptomatology of irritable bowel syndrome after the administration of *L. plantarum CCFM8610* strain. Kageyama et al. (2021) indicated that a *L. plantarum* strain from a Chinese herbal medicine had the potential to protect against coronavirus disease because this strain decreased the levels of interleukin-6 and increased the activation of natural killer cells in the clinical trial. Recently, Kumar et al. (2022a) conducted an *in vivo* study with *Caernorhabditis elegans* and observed that *L. plantarum JBC5* strain could be considered a promising next-generation probiotic that could lead to healthy ageing and enhance longevity in humans. This strain was characterised by reducing oxidative stress and stimulating genes involved in protection against heat damage and pathogenesis, along with stimulating serotonin signalling by increasing cognitive activity.

1.3.2 Metabolism

1.3.2.1 Primary metabolism: microbial cell growth

The transformation of several complex nutrients leads microbial metabolism through a wide range of biochemical reactions to obtain precursor molecules, known as metabolites, to ensure the proper development of the microorganism (Chávarri et al., 2021). The primary metabolism is involved in this process where energy is mainly

obtained from essential nutrients, classified as macronutrients, which high concentration is required for the proper function of the microorganism (Wang et al., 2021c). LAB are considered fastidious microorganisms with specific nutritional conditions to grow (Ayivi et al., 2022). Purines, pyrimidines, amino acids and vitamins are some of the essential growth factors for LAB (Miranda et al., 2021). Therefore, LAB could present proteolytic enzymes to obtain peptides and amino acids, or lipases, to metabolise lipids into useful fatty acids and glycerol to promote microbial growth (Wang et al., 2021c). Nevertheless, the metabolism of carbohydrates is the most important metabolic pathway of LAB because it is the main way to get energy and carbon molecules for microbial growth (Hayek & Ibrahim, 2013).

L. plantarum is considered a highly versatile *Lactobacillus* specie which presents a stronger carbohydrate utilization system compared to another LAB (Corsetti & Gobbetti, 2002). For instance, *L. acidophilus, L. bulgaricus* or *L. helaveticus* are homofermentative LAB using the Embden-Meyerhof-Parnas (EMP) pathway to oxidaze glucose into pyruvate (Bintsis, 2018). Subsequently, the pyruvate molecule is reduced to lactate (homolactic) through the anaerobic catabolic process, where electrons are donated and accepted by organic compounds and an external electron acceptor is not required, known as fermentation (Todorov & de Melo Franco, 2010). Furthermore, some LAB catabolyse glucose through the phosphoketolase pathway obtaining, as a result, carbon dioxide, glyceraldehyde 3-phosphate (GAP) and acetyl phosphate (AcP). Then, GAP goes to EMP pathway producing lactate and, AcP is converted into ethanol (heterolactic) (Khalisanni, 2011). *L. brevis, L. fermentum* or *L. reuteri*, use these pathways to catabolyse glucose, therefore, are classfied as heterofermentative (Bintsis, 2018).

However, *L. plantarum* is a facultative heterofermentative so in the presence of hexoses acts as a homofermentative and, with pentoses, follows the heterolactic pathway (Jung & Lee, 2020). Cui et al. (2021) highlighted that the ability of *L. plantarum* to metabolise different kinds of carbohydrates, such as monosaccharides, disaccharides, sugar alcohol, oligosaccharides, and polysaccharides, was directly correlated to the signal transduction system known as a two-component system that could regulate several physiological processes and the microbial metabolism. Therefore, the high yield of two-component systems in *L. plantarum* has been directly related to its survivability and ability to metabolise a great amount of carbohydrates.

According to the isolation source, the metabolism and the potential beneficial health effects of *L. plantarum* strains could be different. Surve et al. (2022) isolated two *L. plantarum* strains from different Indian food. After a phenotypic characterisation, a great variation in cell adhesion was observed between both strains, as well as a strong difference in sugar metabolism. In this regard, one of the strains had glucansucrase and fructansucrase genes, which are not commonly found in *L. plantarum*. Furthermore, Yu et al. (2021) evaluated thirteen *L.plantarum* strains isolated from different sources such as tomato, cactus fruit, olives or fermented wheat. The results indicated that the carbohydrate metabolism and the stress tolerance of these strains obtained from similar sources did not strongly change. For instance, the strains isolated from tomato and olives in brine were more resistant against acidic pH and salty medium. The authors suggested that the high adaptability of the strains could be related to the variety of mechanisms involved in protecting against stressful conditions. Papadimitriou et al. (2016) studied in depth all the physiological protective mechanisms of LAB against acidic environments, osmotic pressure, high concentration of metals or starvation. For instance, amino acids catabolism is an essential mechanism to preserve the internal pH and reduce energy and stress in LAB (Fernández & Zúñiga, 2006; Guan & Liu, 2020).

1.3.2.2 Secondary metabolism: postbiotic metabolites

Secondary metabolites are commonly synthesised in the late growth phase of the microorganism. Although these metabolites are not indispensable for growing, they could play a key role as defensive or signalling molecules (Thirumurugan et al., 2018). During the last decade, probiotic secondary metabolites have gained importance because they are bioactive functional metabolites producing several beneficial health effects (Chávarri et al., 2021; Mora-Villalobos et al., 2020). Initially, researchers defined these bioactive substances as metabiotics, postbiotic, pharmacobiotics, cell-free supernatants or non-viable probiotics, considering that metabolites, signalling molecules or structural parts of probiotics could be introduced into this classification (Sharma & Shukla, 2016; Singh et al., 2018). Finally, due to the increase in the use of these terms, the definition has evolved indicating that the non-viable probiotics or any other cell lysis components such as polysaccharides, peptidoglycans, teichoic acid or membrane proteins should be defined as parabiotic (Nataraj et al., 2020). Currently, postbiotic are considered bioactive metabolites or other probiotic compounds released during fermentative processes (Abdelazez et al., 2022; Dueñas & López, 2022; Kim et al., 2022) . Chávarri et al. (2021)

emphasized the importance of a wide variety of postbiotic metabolites classified according to their molecular nature (Figure 2). Several organic compounds are included into the postbiotic classification, such as SCFA, polyamines, enzymes, vitamins, bacteriocins, neurotransmitters, amino acids, or proteins. In thos regard, Kareem and Razavi, (2020) reported a group of antimicrobial peptides, known as plantaricins, mainly produced by *L. plantarum* strains useful as food preservatives and a promising future alternative for antibiotic treatments. Li et al. (2022) highlighted that *L. plantarum WSJ-06* strain increased the synthesis of beneficial metabolites like serotonin, vitamin B12 or several organic acids that could alleviate neurological disorders in humans.

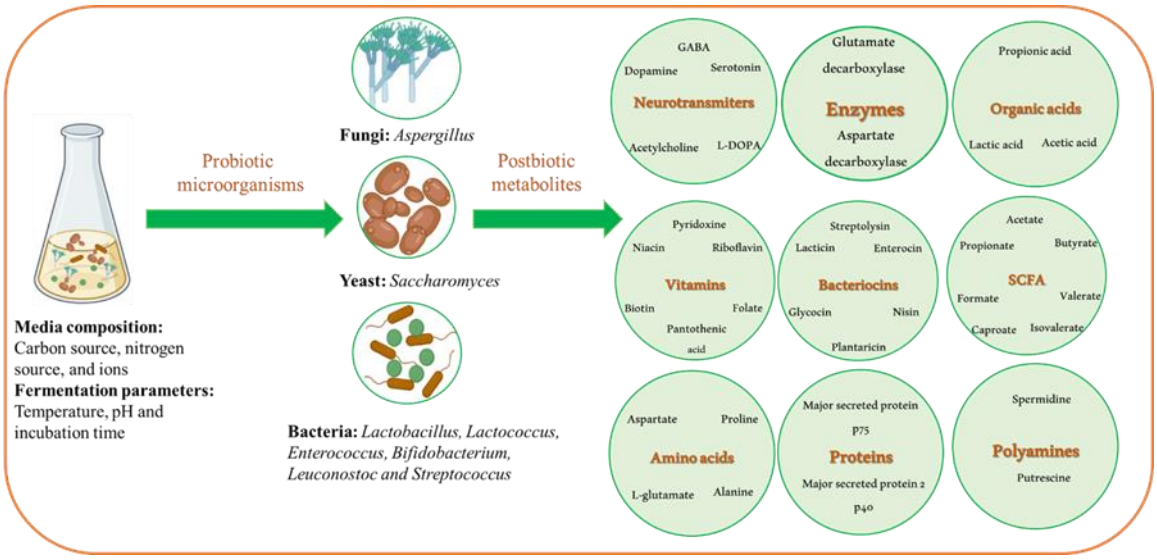


Figure 2: Diversity of postbiotic metabolites synthesized by probiotics (Image adapted from our book chapter Chávarri et al., 2021). GABA: gamma-aminobutyric acid; L-DOPA: L-3,4 dihidroxifenilalanina; SCFA: short chain fatty acids

Giri and Sharma (2022) highlighted the importance of neuroactive metabolites produced by probiotic strains, known as psychobiotics. These compounds can promote the human health by stimulating the central nervous system, acting as neurotransmitters, neurohormones and neuromodulators. Several studies have reported that psychobiotics can produced interesting neurotransmitters that play a key role in human health. For example, Ali and Haq (2010) reported that *A. oryzae* performed the oxidation of tyrosine to enhance the synthesis of 3,4-dihydroxy L-phenylalanine (L-DOPA), an essential neurotransmitter against Parkinson´s disease. *L. lactis* strains also transformed L-DOPA to obtain the neurotransmitter dopamine (Vodolazov et al., 2018). Other species, such as *L. plantarum* or *Streptococcus thermophilus* could synthesise serotonin from the

metabolism of tryptophan (Liang et al., 2019). Currently, the neurotransmitter GABA widely produced by psyschobiotics, has been gaining importance for the last decades due to the wide variety of beneficial effects it can confer on human health (Diez-Gutiérrez et al., 2020).

1.4. Gamma-aminobutyric acid

GABA is a four-carbon non-proteinic amino acid extensively found in eukaryotes and prokaryotes. In 1949, GABA was first discovered in potato tubers (*Solanum tuberosum*). Its production in the plant was directly related to stressful biotic or abiotic situations such as acidification, cold shock, hypoxia or lack of water (Li et al., 2021). One year later, GABA was found in mammalians brain and classified as the most crucial inhibitory neurotransmitter in the central nervous system (Smart & Stephenson, 2019; Spiering, 2018). The GABAergic receptor system presents three central receptors named $GABA_a$, $GABA_b$ and $GABA_c$. This system modulates human behaviours such as mood, sleep and memory (Wang et al., 2021a). Also, this neurotransmitter plays an essential role in preserving health and preventing the development of disorders related, e.g., to the cardiovascular, nervous or endocrinological system (Chávarri et al., 2021).

According to the importance of GABA in human health, the presence of this compound in bacteria, fungi, plants and animals began to be widely studied for the last decades (Ramos-Ruiz et al., 2018). The potential high functionality of GABA attracted the attention of food, pharmaceutical, agricultural and chemical engineering industries. In this regard, Pham et al. (2016) reported that GABA was an interesting molecule to produce bioplastics, as this amino acid is the precursor of pyrrolidone, the main monomer required for synthesizing the biodegradable commercial polymer Nylon 4. Furthermore, Liu et al. (2015) proposed that GABA could be a good choice for acid mine drainage bioremediation due to the protective effect of this molecule against acidic environments. On the other hand, the food and pharmaceutical industries have focused on developing food supplements and healthier fermented foods (Boonstra et al., 2015; Champagne et al., 2018).

Currently, GABA can be chemically synthesized or obtained by biological processes (Dhakal et al., 2012). The chemical synthesis of GABA follows the Hell-Volhard-Zelinsky method, which is a simple, reliable process. However, this chemical synthesis has low efficiency and a high environmental impact because a lot of energy and

chemicals are required (Wang et al., 2016). Hence, GABA production has moved to use biological processes such as plants or microorganisms. For instance, plants can accumulate GABA under stressful conditions, but the inefficiency of the process and high cost prevent it from being scalable at industrial level (Li et al., 2021). Consequently, the production of GABA by microorganisms has gained importance due to its high efficiency, affordable cost and low impact in the environment (Sarasa et al., 2019). Figure 3 draws the biosynthetic pathways that microorganisms can use to produce GABA. The machinery to produce GABA depends on the type of microorganisms.The putrescine pathway (Figure 3a) is often used by *Escherichia coli* or the fungi *Aspergillus oryzae* (Akasaka et al., 2018; Cha et al., 2014). Glutamic acid decarboxylase (GAD) pathway (Figure 3b) is more extended among probiotic microorganisms such as *Lactobacillus* and *Bifidobacterium* species (Kim et al., 2014; Yunes et al., 2020).

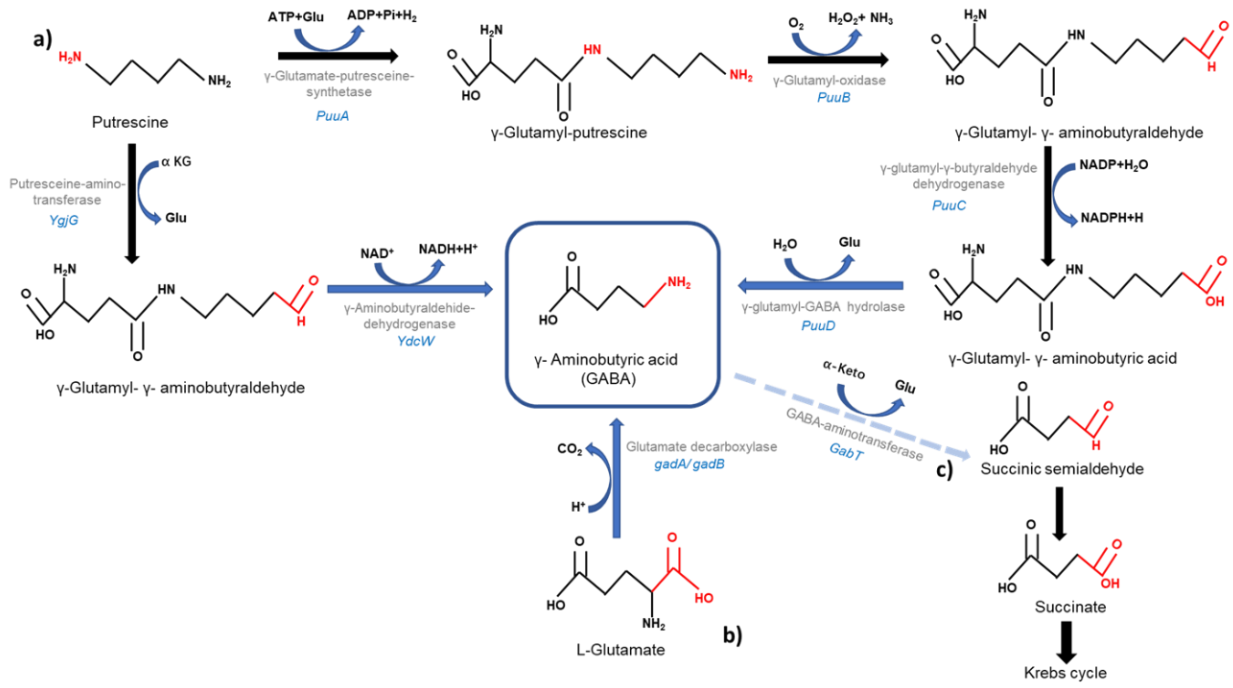


Figure 3: Biosynhtetic pathways that microorganisms can use to produce gamma-amino butyric acid: a) Putrescine pathway; b) Glutamic acid decarboxylation pathway; c) Degradation process of gamma-amino butyric acid (Image obtained from our article Diez-Gutiérrez et al., 2020; Annex I.I).

Furthermore, *L. plantarum* species synthesize GABA activating the GAD pathway under stressful environments (Phuengjayaem et al., 2021). An acidic environment activates the GAD pathway (Figure 4), which begins with introducing a molecule of L-glutamic acid (L-Glu) into the cell. This molecule is introduced into the

cell using an electrogenic antiporter, codified by a *gadC* gene, which is also involved in the exportation of the synthesized GABA molecule (Yunes et al., 2016). When L-Glu is inside the cell, it is decarboxylated by the GAD enzyme encoded by the *gadB* gene, obtaining one GABA molecule and, in consequence, the cytoplasmic pH increases consuming one proton and releasing one carbon dioxide molecule. Afterwards, GABA is pumped to the extracellular matrix coupled with the introduction of another L-Glu molecule which enhances the production of proton motive force and the accumulation of adenosine triphosphate (ATP) (Papadimitriou et al., 2016). Furthermore, under energy requirements GABA can be degraded into succinic semialdehyde using a GABA-aminotransferase enzyme codified by a *gabT* gene, and then, succinic semialdehyde can be converted into succinate been catalyzed by succinic semialdehyde dehydrogenase encoded by a *gabB* gene. Finally, the succinate molecule enters into the tricarboxylic acid cycle (Sarasa et al., 2019). GABA synthesis is strain-dependent and the efficiency of the GAD pathway is related to different parameters that may influence the expression of the *gad* genes and ultimately the yield of the process. Some of the parameters studied have been fermentation temperature, media pH and concentration of nutrient precursors of carbon and nitrogen sources for microorganisms (Chávarri et al., 2021; Diez-Gutiérrez et al., 2020).

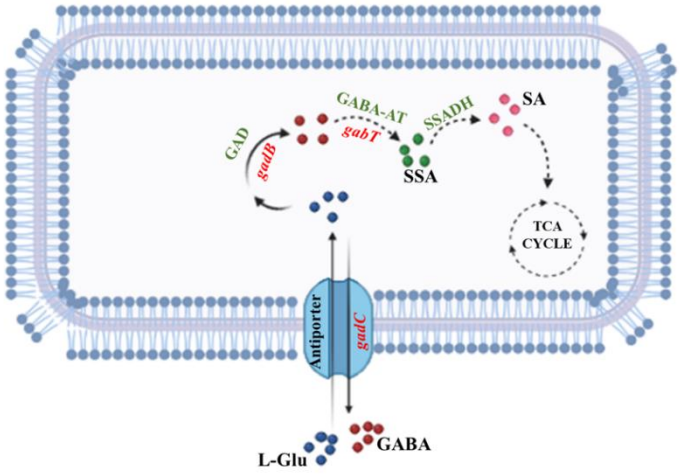


Figure 4: Representation of the glutamic acid decarboxylase pathway (GAD). L-glu: Glutamic acid, GABA: gamma-aminobutyric acid, *gadC:* antiporter gene, GAD: glutamic acid decarboxylase enzyme, *gadB:* GAD gene, GABA-AT: GABA-aminotransferase, *gabT:* GABA-aminotransferase gene, SSA: succinic semialdehyde, SSADH: succinic semialdehyde dehydrogenase, SA: succinic acid, TCA: tricarboxylic acid

1.5. Biotechnological processes for probiotics and postbiotics production

The wide therapeutic effect of probiotics has opened a worldwide market focused on developing and distributing new agri-food and pharmaceutical products that have a specific effect on human health and could improve people's quality of life. The scaling of the biotechnological process for the production of probiotics needs to be adjusted to the microorganism and the end product (postbiotic) that is going to be developed and launched into the market (Peter et al., 2022). The biotechnological process is firstly focused on the isolation and characterisation of the most suitable probiotic microorganism. Therefore, selecting adequate strains involves identification, evaluation of growth, *in vitro* and *in vivo* assessment of probiotic capacity such as adhesion, resistance against gastrointestinal conditions, production of bioactive compounds and activity against pathogens (Aguirre Rodriguez & Hernán Moreno Cardozo, 2012). Afterwards, the growth kinetics is studied focusing on the production of the highest biomass yield, which is directly linked to the culture media composition. Therefore, it is essential to select and design a proper culture media adjusted to the nutritional requirements of each type of microorganism and certain parameters of the fermentation process to reach the exponential growth phase and the greatest microbial cell growth in less time (Fenster et al., 2019).

Generally, LAB as *L. plantarum,* are grown in specific high-nutrient media known as Man Rogosa Sharpe (MRS) which is composed of minerals, carbon and nitrogen sources to ensure adequate microbial growth. However, the synthetic media used are expensive, increasing the expenses for the scale-up fermentation process (Kumar et al., 2022b). Therefore, natural fermentation substrates, such as agri-food by-products, have been proposed as a low-cost nutritious source (Freire-Almeida & Maldonado-Alvarado, 2022). At the same time, reusing these agri-food by-products is an excellent way to promote the circular economy and follow the FAO guidelines, where companies were encouraged to reduce food waste, environmental impact and money lost (Alves de Castro et al., 2020). Pepper seeds (Cvetković et al., 2022), cheese whey (Raho et al., 2020), apple (Mnisi et al., 2022), tomato (Szabo et al., 2018), guayaba (Casarotti et al., 2018) or orange (Alves de Castro et al., 2020) by-products are some of by-products used as fermentation substrates. Furthermore, Mármol et al. (2021) highlighted that some by-products, like fruits and vegetables, were good sources of bioactive compounds. Hence, the composition of by-products could be helpful to complete the extensive promoting health effects of *L.*

plantarum and help to develop a more nutritious final ingredient (Darby & Jones, 2017). Likewise, postbiotic metabolites could be produced using agri-food by-products. In this regard, Sharma et al. (2021a) developed a biotechnological process for producing GABA and lactic acid by *L. plantarum LP-9* strain using a bran by-product. The same authors (Sharma et al., 2021b) successfully achieved the production of lactic acid and plantaricin by developing an economic fermentation media by using whey permeate and palmyra palm sugar as carbon sources and whey protein hydrolysate as nitrogen sources.

According to the main objective of any fermentation process, biomass production or synthesis of bioactive metabolites, the nutritional profile of the growing medium needs to be adjusted to get the maximum process yield (Fenster et al., 2019). The optimum conditions can be influenced by other fermentation parameters that need also to be optimised such as temperature, pH, oxygenation or agitation, which are industrially controlled and standardised using bioreactors (Lacroix & Yildirim, 2007).

After the fermentation, the next step in the manufacturing process is focused on the recovery and storage of the cell biomass and bioactive compounds produced. One of the critical points in this step is the adverse conditions that these sensitive microorganisms and their bioactive compounds are subjected such as moisture, temperature and/or osmotic stress. Furthermore, probiotics need to be administered in a concentration higher than 10^6 CFU/mL to confer beneficial health effects in the host (Afzaal et al., 2019). However, most of probiotics are sensitive to the environmental conditions of the gastrointestinal tract, such as acid pH and high concentrations of bile salts that can affect the viability of probiotics (Shori, 2017). Selecting resistant probiotic strains, conditioning strains to stressful situations, genetic manipulation, or microencapsulation techniques are some of the solutions commonly used to address this problem (De Prisco & Mauriello, 2016).

Microencapsulation is a widely used technology mainly focused on creating a semi-permeable spherical capsule ranging in size from several microns to one millimetre. Capsules not only confer a protective layer for the probiotic, as it is also useful to manage, store and control the probiotic release (Rokka & Rantamäki, 2010). Different encapsulation materials could be used depending on the specifications of the final product, and they are classified as safe ingredients for use in the food industry (Shori, 2017). Among the best-known encapsulation materials, there are polymers of different chemical structures from plants (starch or pectin), animals (milk protein, chitosan, or

gelatine) or algae (alginate or agar) (Rathore et al., 2013). Alginate is one of the most common biopolymer used for encapsulation processes. This biopolymer can be extracted from brown algae of the genus *Laminaria, Macrocystis* or *Ascophyllum,* or it can even be produced by bacteria of the genus *Pseudomonas* or *Azotobacter* (Hassan et al., 2020). The functionality of the alginate will be affected by its extraction process, because it will determine the proportion and structure of the two acids that compose this biopolymer. The adhesion capacity of alginate will be close related to its composition and will determine its ability to create the microcapsules structure (Wandrey et al., 2010)

The production of capsules can be performed using different encapsulation techniques such as extrusion, emulsion, fluid bed, freeze-drying, spray drying, hybridization technologies or electrospinning (Martín et al., 2015).Some of these techniques have been used to get functional ingredients composed of probiotics and the postbiotic metabolite GABA, using different types of biopolymers. For instance, Ma et al. (2020) used *L. brevis TCCC 13007* to produce GABA through fermentation techniques and a functional ingredient, composed of fermented broth enriched with GABA and maltodextrin, was produced using spray drying techniques. Misra & Mishra (2022) and Pandey & Mishra (2021) also used spray drying techniques to develop a functional powder to be used in different food formulations. In these studies, the ingredient was composed of *L. lactis SKL 13* or *L. plantarum* and GABA encapsulated using maltodextrin, inulin and dextran. Furthermore, Pandey et al. (2021) also encapsulated LAB and GABA for food formulations through ultrasonication techniques where double emulsion microcapsules were developed using dextran and whey protein.

The election of the best technique depends on the microorganisms size and its ability to survive under the encapsulation process and storage conditions. Also, the encapsulation technique selected is related to the viscosity, density, or the addition of prebiotic and/or bioactive compounds. Extrusion is a cheap and simple encapsulation technique harmless for probiotics that enhances microorganisms viability (Altamirano-Ríos et al., 2022). In this case, the probiotic is mixed with an encapsulation material, usually a wall material or polymer such as alginate, which is passed through a nozzle, producing droplets. These droplets should fall into a hardening bath composed of gelation or a crosslinking agent like calcium chloride (Sultana et al., 2022). Chávarri et al. (2012) explained that the jet speed can divide the extrusion method into dropwise (gravity, coaxial flow and electrostatic potential) and jet breakage (vibration mechanism, cutting

method and centrifugal strength). The development of the best capsule using extrusion techniques is related to different physicochemical conditions. Historically, several scientists have studied the theoretical explanation for controlling droplet formation by liquid extrusion through a nozzle. Heinzen et al. (2004) indicated that the capsules' structure and size depend on the extrusion velocity, surface tension, friction and gravitational force. Whelehan & Marison (2011) highlighted that it is important to achieve the optimal conditions to produce equal-sized droplets for further scale them industrially.

After encapsulation process, a drying step is also required to ensure the viability of the probiotic during handling, storage and transport (Shu et al., 2018). Among the possible drying techniques, lyophilization stands out eliminating water through an initial freezing process followed by a vacuum phase (Acosta-Piantini et al., 2019). However, the viability of the probiotic could be affected through the lyophilization process, making essential to add a cryoprotective agent during or after the encapsulation (Halim et al., 2017). Some of the most common cryoprotectants are glycerol, betaine, glucose, sucrose, powdered milk, or other different type of polymers (Jalali et al., 2012).

The end-product of this biotechnological process gives as a result functional ingredients, where probiotics and postbiotic have received all the attention from the industry due to their physiological effects (Aguilar-Toalá et al., 2018; Syngai et al., 2016). Currently, these ingredients are mainly consumed orally as part of functional foods, drugs or dietary supplements (Yoha et al., 2022).

The global market of functional ingredients has focused on probiotics and postbiotic compounds. Grand View Research (2022) reported that in 2021 the probiotic market had a profit of around 58.17 billion dollars, and it is forecasted to grow at a compound annual growth rate of 7.5% up to 2030. Kerry Foods (2022) have reported that Asia, specifically China, holds the largest market share of probiotics followed by Europe. Among probiotics, *Lactobacillus spp* is the most commonly used, and in 2021 reached the highest market share (The Insight Partners, 2022). Likewise, the postbiotic market share is also experiencing an increase in attention as the global market size of GABA was around 85 million dollars in 2018, and is expected to reach 126 million by 2032 with a compound annual growth rate of 4.5% (Einpresswire, 2022). In addition, Koe (2022) recently highlighted that GABA was the wider functional ingredient used in Japan in 2021 as a supplement to reduce stress and blood pressure, and enhance sleep.

2. HYPOTHESIS AND OBJECTIVES

This research aimed to design a microencapsulated functional ingredient enriched with *L. plantarum K16* and the postbiotic metabolite GABA obtained through fermentation processes. The hypothesis of this Ph.D. Thesis puts forward the idea that for the development of a new functional ingredient based on a probiotic performance, it is necessary to assess its safety and ability to produce a sufficient amount of postbiotic, and to optimise the fermentation conditions. Also, the use of an agri-food by-product as a culture medium is a very interesting alternative for economic and environmental reasons. Finally, the hypothesis states that the new functional ingredient must ensure the efficacy of the probiotic and postbiotic action in the intestine, which requires the resistance and stability of both against gastrointestinal conditions, and that the microencapsulation technique is a suitable methodology to achieve this.

To this end, the specific objectives of this PhD project were to:

1. Identify and isolate a LAB from natural kimchi that presents the ability to synthesize the postbiotic metabolite GABA through fermentation processes.
2. Evaluate the safety and the probiotic capacity of the selected LAB strain through *in vitro* assays.
3. Determine the optimal fermentation conditions for the selected LAB strain to produce GABA using a commercial culture medium.
4. Identify a culture medium from agri-food by-products as a fermentation substrate for the selected LAB strain to enhance microbial cell growth and synthesize GABA.
5. Design a novel functional ingredient using microencapsulation technologies to guarantee the protection of the selected strain and GABA against gastrointestinal conditions and ensure their release in the gut.

3. MATERIALS AND METHODS

3.1. Isolation of *Lactiplantibacillus plantarum K16* from natural kimchi

This research was carried out using a *Lactiplantibacillus plantarum K16* strain, which presented slightly white, circular, creamy colonies in MRS agar (Figure 5), isolated from the fermented food known as kimchi. For the isolation process, kimchi was prepared with cabbage and preincubated overnight in water supplemented with 25.5 g/L of sodium chloride. Then, the cabbage was fermented in 400 mL of sterilised distilled water supplemented with garlic, sodium chloride and glucose. Samples were taken after 24 h of incubation, and serial dilutions were prepared and plated on MRS agar (Sigma, Misuri, USA). The colonies that grew in MRS after 48 h of incubation at 37 ºC and 5% of carbon dioxide were selected morphologically and individually isolated to proceed with the identification process.

Figure 5: *Lactiplantibacillus plantarum K16* colonies in MRS agar

3.1.1 Identification of lactic acid bacteria

The first step in carrying out the identification of LAB was the detection of lactic acid production. Therefore, petri plates of MRS supplemented with 0.3% calcium carbonate were used to grow the isolated bacteria at 37 ºC and 5% carbon dioxide for 48 h (Figure 6a). The bacteria that produced lactic acid enhanced the solubility of the calcium carbonate, creating a clear zone (Figure 6a.1), considering those strains as possible LAB strains. Hence, the catalase test (Figure 6b) and Gram staining(Figure 6c) were performed to continue the isolation process. Catalase-negative (Figure 6b.2) and Gram-positive (Figure 6c.2) bacteria were considered LAB (Monika et al., 2017).

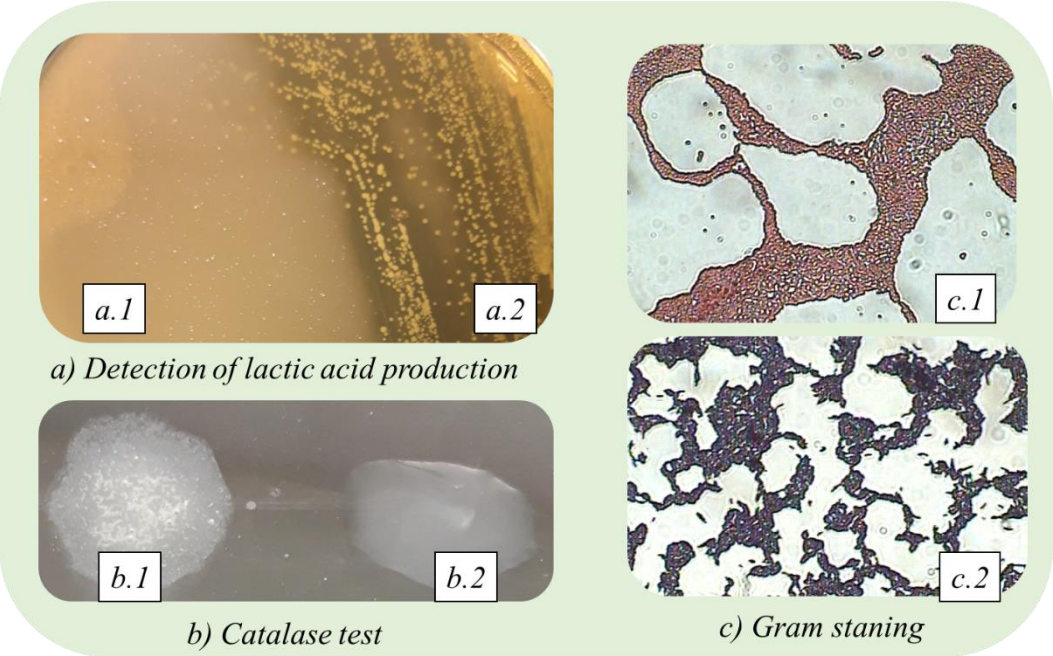


Figure 6: Test to identify lactic acid bacteria: a) Detection of lactic acid bacteria test, showing positive lactic acid production (a.1) and negative acid production (a.2); b) Catalase test, showing catalase positive (b.1) and catalase negative (b.2); c) Gram staning, presenting gram negative strains (c.1) and gram positive strains (c.2)

3.1.2 Assessment of gamma-aminobutyric acid production

The strains identified as LAB were finally grown to detect their ability to synthesize GABA. In this case, the microorganisms were inoculated in MRS broth (Sigma) supplemented with 1% of L-Glu (Scharlab,Barcelona, Spain) and incubated at 37 ºC for 48 h. Afterwards, supernatants were collected, centrifuged at 12,000 rpm for 5 min and passed through a 0.22 µm filter of polyethersulfone. The production of GABA was quantified using Ultra-High Performance Liquid Chromatography (UPLC) coupled with Mass Spectrometry (MS) detection. An ultra-ACQUITY UPLC H-class system (Waters, Milford, USA) with a HILIC column (130 Å pore size; 1.7µm particle size; 2.1 mm internal diameter; 100 mm length) (Waters) coupled with a SecurityGuard ULTRA cartridge pre-column (Waters). Column temperature was set to 30 °C, sample temperature was set to 10 °C, and injection volume was 3 µL. An isocratic elution with a mixed-in volume of 5% of acetonitrile (HPLC grade, Scharlab) and 95% of 0.1% formic acid (LC-MS grade, Scharlab) prepared in Milli-Q water as mobile phase, with a flow rate of 0.25 mL/min, was used. A triple quadrupole MS equipped with an orthogonal electrospray ionisation source (ACQUITY TQD, Waters) was used for detection. The instrument was operated in positive mode electrospray, and MS settings were used as follows: capillary

voltage 3.05 kV, desolvation temperature 400 °C, source temperature 120 °C, cone and desolvation gas (nitrogen) flow 60 L/h and 800 L/h, respectively, and collision gas (argon) flow 0.10 mL/min. High-purity nitrogen and argon were used (Nippon Gases, Madrid, Spain). MS was run in multiple reaction monitoring mode, including two ion transitions for GABA: m/z 104>87 for quantification and m/z 104>69 for identification. Data acquisition and quantification were performed using MassLynx software version 4.1 (Waters). Quantification was performed against a linear (1/x weighted) regression curve based on duplicate calibration GABA standard solutions injections. The results showed that only one isolated LAB strain seemed to synthesize GABA, further sequenced and identified as *Lactiplantibacillus plantarum K16.*

3.1.3 Microbial growth in commercial broth

Microbial growth kintetics was evaluated to determine the potential of *L. plantarum K16* strain to achieve enough biomass for production to be economically profitable. Therefore, a timeline analysis of the microbial cell growth was assessed by inoculating 1% of *L. plantarum K16* in MRS broth and incubated at 37 ºC for 72 h. Samples were taken after 0, 2, 4, 24, 48 and 72 h of fermentation. The growth was measured by plating serial dilutions in MRS agar and counting colonies to calculate the colony-forming units (CFU) and expressed as log CFU/mL (± 0.01). A Crison Basic 20 pHmeter (Crison, Barcelona, Spain) was used to determine the pH value (± 0.1) of ther fermentation media, and the concentration of glucose was determined using a Quantofix refractometer (Macherey-Nagel, Düren, Germany) . The CFU/mL values were used to calculate the specific microbial growth rate after 24 h. The comsuption of glucose and the CFU/g were used to calculate the biomass yield.

3.2. Characterisation of *Lactiplantibacillus plantarum K16*

The characterisation process of *L. plantarum K16* was focused on the biochemical profile of the strain by studying carbohydrates metabolism and its enzymatic activity. Likewise, a safety assessment was based on the haemolytic activity and the susceptibility of *L. plantarum K16* against several antibiotics. Furthermore, *in vitro* antimicrobial studies were carried out to determine the potential of this strain to inhibit the growth of human pathogens.

3.2.1 Safety evaluation of *Lactiplantibacillus plantarum K16*

3.2.1.1 Antibiotic susceptibility

The disk-diffusion antibiotic susceptibility test was used to evaluate the antibiotic resistance of *L. plantarum K16* strain according to the procedure used by Dowarah et al. (2018) and Diez-Gutiérrez et al. (2022). This procedure is explained in detail in the research article included in Annex I.II. In this regard, 10 mL of MRS broth was used to inoculate one colony of *L. plantarum K16* and grow it overnight at 37 ºC. Then, a swab was used to spread the bacteria uniformly through MRS plates, sterilised tweezers were used to put the disk on the agar and, after that, plates were incubated at 37 ºC for 48 h. The length of the diameter of the inhibition zone was measured in millimetres (± 0.1) for all antibiotics and, according to the size, the bacteria was considered susceptible (≥ 21 mm), intermediate (16-20 mm) or resistant (≤ 15 mm) to the antibiotic (Dowarah et al., 2018).

3.2.1.2 Haemolytic activity

The haemolytic activity of *L. plantarum K16* was tested as previously described (Angmo et al., 2016). Briefly, Columbia blood agar plates (Scharlab, Barcelona, Spain) were enriched with 5% sheep blood to grow the microorganism at 37 °C for 48 h. The haemolytic activity was considered positive when the plates observed a halo.

3.2.2 Probiotic ability of *Lactiplantibacillus plantarum K16*

3.2.2.1 Carbohydrates metabolism

Analytical Profile Index (API) 50CHL kit was performed according to the procedure defined by the manufacturer (Biomerieux, Marcy-l´Eloile, France) and the results obtained were analysed using the API web (apiweb.biomerieux.com). This procedure is explained in detail in the research article included in Annex I.II.

3.2.2.2 Enzymatic profiling

The enzymatic activity of *L. plantarum K16* was determined using the API ZYM kit (APISystem), which was used according to the procedure defined by the manufacturer (Biomereux). Hence, the colour intensity was related to the enzymatic activity and expressed as nmol of the substrate. This procedure is explained in detail in the research article incuded in Annex I.II.

3.2.2.3 Antimicrobial activity

The antimicrobial effect of *L. plantarum K16* was tested using the agar disk-diffusion method and the agar well-diffusion method. The antimicrobial effect using the agar disk-diffusion method was performed as previously described (Abedi et al., 2013). In this case, the biomass and the supernatant of *L. plantarum K16* were used against common pathogens such as *Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes.* Furthermore, the agar well-diffusion method was used as previously described (Balouiri et al., 2016) to evaluate the antimicrobial effect of *L. plantarum K16* against the biomass of pathogens.

3.2.2.4 *In vitro* studies with cell culture lines

The human colon adenocarcinoma (Caco2) cell line (Figure 7) was purchased from Sigma. This cell line was cultured using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% of fetal bovine serum (Sigma), 1% of non-essential amino acids (Cytiva, Emeryville, USA), 2% of bicarbonate (Cytiva) and 0.63% of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Cytiva).

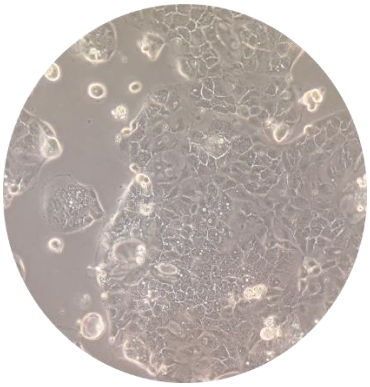


Figure 7: Photograph of Caco2 cells using inverted optical microscope

Caco2 cells were used to evaluate the adhesion ability of *L. plantarum K16* strain and its ability to interact with common pathogenic bacteria, such as *E. coli, S. typhimurium* and *L. monocytogenes.* For that purpose, Caco2 were grown following the method described by Yu et al. (2013). The study was performed using 48-well culture plates which were filled with 10^4 cells *per* well, and the culture media was changed every three times *per* week.

A stable monolayer was created after 21 days, and experiments were carried out according to the method described by Jamyuang et al. (2019) with slight modifications. *L. plantarum K16* was grown overnight in MRS broth, centrifuged at 12,000 rpm for 15

min and resuspended in culture media, getting a concentration of 9 log CFU/mL. Likewise, the pathogenic bacteria were grown overnight in Brain Heart Infusion broth, centrifuged at the same conditions and resuspended in culture media, getting a concentration of 9 log CFU/mL.

Before adding bacteria, the cultivation media was removed, and the monolayer was washed twice with Phosphate Buffer Saline. The ability of *L. plantarum K16* to inhibit the adhesion of pathogens was determined by comparing the pathogen adhesion with and without the presence of *L. plantarum K16.* Hence, the following experiments were performed (Figure 8):

- The individual adhesion of each bacterium, used as a control, was evaluated by adding 300 µL of each bacterium alone (*L. plantarum K16, E. coli, S. typhimurium* and *L. monocytogenes*) and incubating with the cells for 1 h at 37 ºC with 5% carbon dioxide.
- A protective assay was performed by adding 150 µL of *L. plantarum K16* into the Caco2 cell monolayer, incubating for 30 min at 37 ºC with 5 % carbon dioxide and, independently, 150 µl of pathogenic bacteria were added and incubated for 30 min in the same conditions.
- A displacement of pathogen bacteria was assessed by independently adding 150 µL of each pathogen incubated for 30 min at 37 ºC with 5 % carbon dioxide. Then 150 µL of *L. plantarum K16* were added and incubated for another 30 min in the same conditions.
- The competitive exclusion was evaluated by adding 150 µL of *L. plantarum K16* at the same time as 150 µL of a pathogenic bacterium and incubating for 1 h.

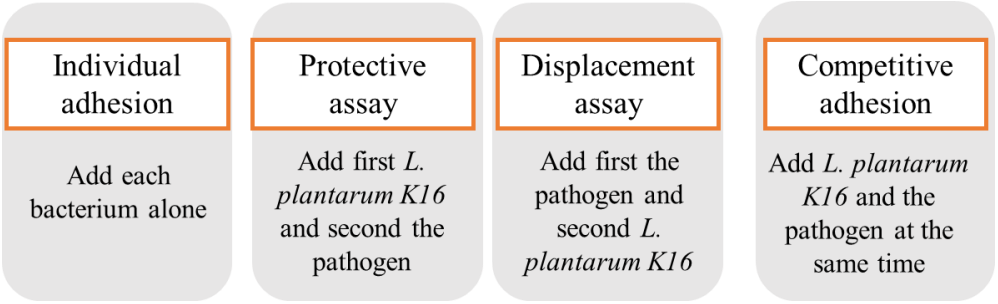


Figure 8: Experimental procedures used for the studies of adhesion, protection, displacement and competition of *L. plantarum K16* against *E. coli, S. typhimurium* and *L. monocytogenes* pathogenes.

In all the experiments, after the incubation time, the monolayer was washed 3 times with Phosphate Buffer Saline to remove the unattached bacteria, and the cells were lysed with 0.1% of Triton X-100 for 10 min. From Caco2 cells lysed, adhered bacteria were counted by diluting and plating in selective agar *per* each microorganism. Therefore, *L. plantarum K16* strain were counted in MRS agar, *E. coli* in Eosin Methylene blue agar (Scharlab), *S. typhimurium* in Xylose lysine tergitol agar (Scharlab) and *L. monocytogenes* in Listeria selective agar (Scharlab).

3.3. Gamma-aminobutyric acid production

The production of GABA by *L. plantarum K16* was initially assessed in MRS broth to determine the main parameters that modulate the synthesis of this postbiotic metabolite and achieve the highest yield in this medium. The experimental design using MRS broth is explained in detail in the research articles included in Annexes I.II and I.III. Then, a fermentation trial was conducted with different agri-food by-products to choose the most suitable fermentation substrate in order to develop the functional ingredient. The experimental methodology used to assess the ability of agri-food by-products to be used as fermentation substrates is explained in detail in the research article included in Annex I.III.

3.3.1 Gamma-aminobityric acid production using commercial broth

The optimisation of the GABA production by *L. plantarum K16* using MRS broth was performed using an one-factor-at-a-time (OFAT) experimental design. Several stages were carried out by evaluating different levels of one fermentation parameter while the other fermentation parameters were kept fixed. The scheme of the OFAT performed is shown in Figure 9.The fermentation parameters involved in the optimisation study were incubation temperature, yeast extract concentration, fermentation time, percentage of inoculum, initial pH, and concentration of monosodium glutamate (MSG) and glucose. For each fermentation trial, the amount of GABA produced (mg/L; ± 0.01), the colonies counting and the pH were measured. The amount of GABA was determined by UHPLC-MS as previously described in 3.1.2 subsection. The microbial growth of *L. plantarum K16* during the fermentation process was assessed by plating serial dilutions in MRS agar and colonies counting to calculate the CFU and expressed as log CFU/mL (± 0.01).

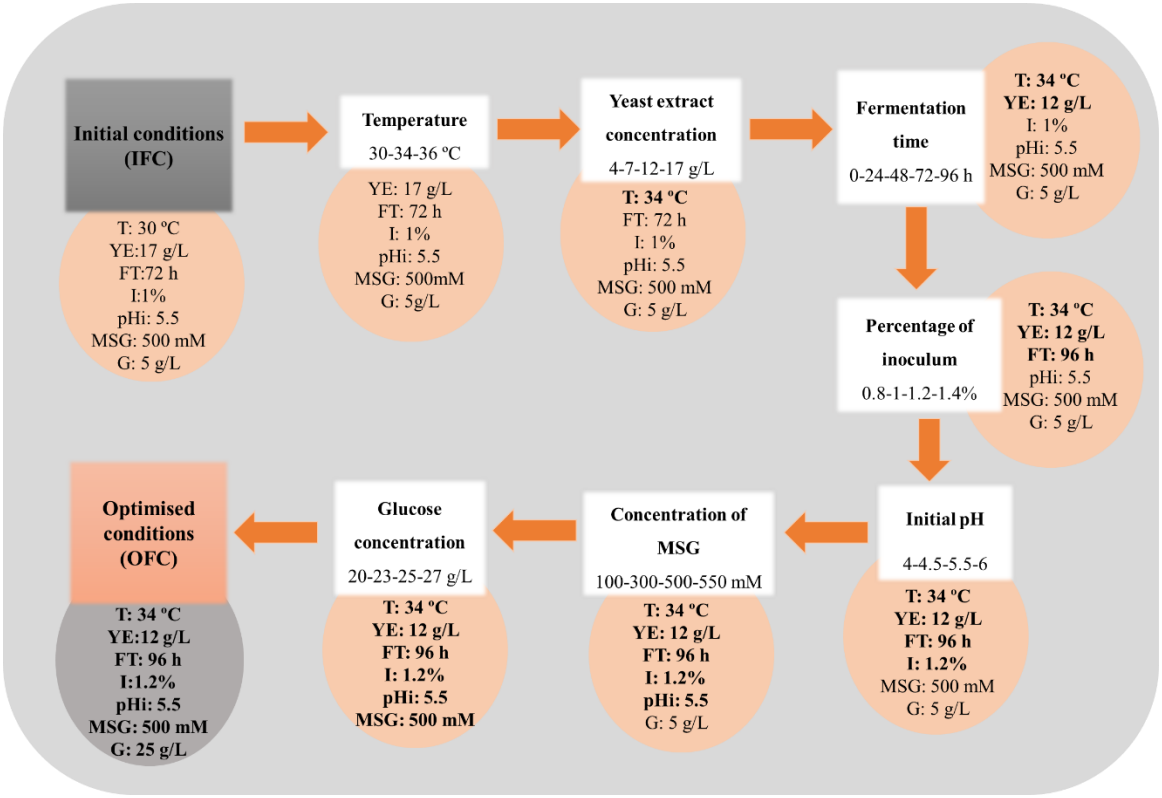


Figure 9: One-factor-at-a-time experimental design to study the factors affecting the production of gamma-aminobutyric acid *by L. plantarum K16* using MRS broth. The progress of the design is highlighted by bolding the studied parameters starting from initial conditions (IFC) to optimised conditions (OFC). T: temperature, YE: yeast extract, FT: fermentation time, I: percentage of inoculum, pHi: initial pH, MSG: monosodium glutamate, G: glucose (G).

3.3.2 Gamma-aminobityric acid production using agri-food by-products

The optimisation of of the GABA production process conducted in MRS broth helped to determine the most important parameters affecting the fermentation by *L. plantarum K16*. Subsequently, fermentation trials were performed to evaluate how different agri-food by-products could be used as fermentation substrates for GABA production. Before the fermentation process, these by-products were dried after reception and storage in sealed vacuum plastic bags in a temperature controlled room (20 ºC).

Figure 10 shows the steps to prepare the fermentation media from the agri-food by-products. The research article included in Annex I.III indicates the nutritional composition of each by-product. As described before for the commercial MRS broth, analytical samples of the fermented media were taken to determine the pH, GABA amount and the log CFU/mL.

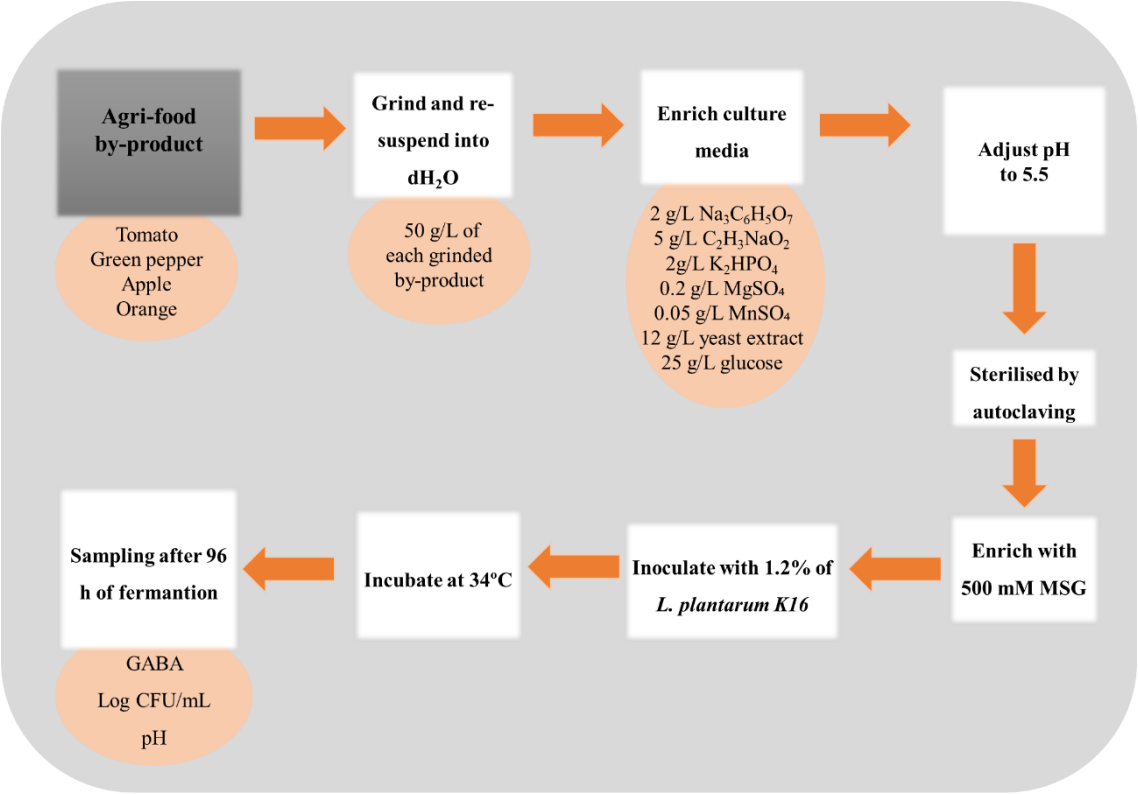


Figure 10: Steps used in the preparation of fermentation media from agri-food by-products for gamma-aminobutyric acid (GABA) production by *L. plantarum K16*

The fermentation trials using green pepper, orange, tomato and apple by-products indicated that they could be used as fermentation substrates for GABA synthesis by *L. plantarum K16* strain. Furthermore, the GABA productions using tomato by-product as a fermentation substrate was the greatest and, therefore, this by-product was chosen to perform an optimisation process of the biomass production and the synthesis of GABA in order to develop the functional ingredient.

3.4. Tomato by-product as fermentation substrate

The results of the fermentation trial indicated that tomato by-product could be considered a suitable option to be used as substrate to develop the functional ingredient. In this regard, a study was performed to determine how combining different nutrients could influence the growth of *L. plantarum K16* and, in consequence the GABA production, in order to obtain the highest biomass and GABA yield.

3.4.1 Evaluation of microbial cell growth using tomato by-product

The microbial cell growth was evaluated according to a Box-Behnken experimental design where the supplementation of different concentrations of glucose,

yeast extract and minerals were assayed using tomato by-product as substrate. In this experimental design, no MSG was supplied to promote the *L. plantarum K16* growth during 24 h. The results will be useful for building a response surface matrix to evaluate the interaction among the three different supplements during the growth of *L. plantarum K16.* Consequently, 15 different experiments with 3 central points were carried out by combining different concentrations of glucose (0, 12.5, and 25 g/L), yeast extract (0, 6, and 12 g/L) and minerals (0, 50, and 100%) (Table 2).

Table 2: Box-Behnken experimental design combining different concentrations of glucose, yeast extract and minerals to evaluate their impact on the growth of *L. plantarum K16* using tomato by-product as fermentation medium.

Experimental unit	***Glucose concentration (g/L)***	***Yeast extract concentration (g/L)***	***Minerals (%)***
1	0	0	50
2	12.5	6	50
3	12.5	12	0
4	25	0	50
5	0	6	100
6	25	6	0
7	12.5	0	0
8	12.5	6	50
9	12.5	6	50
10	12.5	0	100
11	12.5	12	100
12	0	12	50
13	0	6	0
14	25	12	50
15	25	6	100

In the specific case, 100% of minerals is the combination of 2 g/L sodium citrate, 5 g/L sodium acetate trihydrate, 2 g/L dipotassium hydrogen phosphate, 0.2 g/L magnesium sulphate, and 0.05 g/L manganese sulphate. In all the experiments, the fermentation media was adjusted to an initial pH of 5.5. Afterwards, the media was inoculated with 1.2% of *L. plantarum K16* in MRS broth and incubated for 24 h at 34 ºC without shaking. The microbial growth was evaluated by plating serial dilutions to calculate log CFU/mL after 24 h. Box-Behnken experimental design and response surface methodology were performed with JMP PRO 14 statistics software (SAS, Cary,USA).

3.4.2 Evaluation of gamma-aminobutyric acid synthesis using tomato by-product

A Box-Behnken experimental design was also used to evaluate how different concentrations of glucose, yeast extract and MSG could interact and modulate the GABA production using tomato by-product as substrate. The results will help build an response surface matrix to evaluate the interaction among these three compounds for GABA production by *L. plantarum K16.* In this regard, 15 different experiments with 3 central points were carried out by combining different concentrations of glucose (20, 25 and 30 g/L), yeast extract (4, 8 and 12 g/L) and MSG (350, 450 and 550 mM) (Table 3). In all the experiments, the fermentation media were supplemented with 100% minerals, and the initial pH was adjusted to 5.5. Afterwards, the media was inoculated with 1.2% of *L. plantarum K16* in MRS broth and incubated for 96 h. Analytical samples of the fermented medium were taken to determine the GABA concentration. Box-Behnken experimental design and response surface methodology were performed with JMP PRO 14 statistics software (SAS, Cary,USA).

Table 3: Box-Behnken experimental design combining different concentrations of glucose, yeast extract and MSG to evaluate their impact on the GABA synthesis by *L. plantarum K16* using tomato by-product as fermentation medium.

Experimental unit	***Glucose concentration (g/L)***	***Yeast extract concentration (g/L)***	***MSG (mM)***
1	25	12	350
2	20	4	450
3	30	4	450
4	25	4	350
5	30	8	350
6	30	8	550
7	30	12	450
8	25	8	450
9	25	8	450
10	20	8	350
11	25	4	550
12	20	12	450
13	25	12	550
14	20	8	550
15	25	8	450

3.5. Evaluation of resistance against gastrointestinal conditions

The healthy effect of *L. plantarum K16* and that of its postbiotic metabolite GABA will benefit the gut microbiota (Dos Reis Lucena et al., 2021). Therefore, it is essential to ensure that both probiotic and postbiotic can resist extreme ambient conditions, such as low pH and high salt concentration, present in the gastrointestinal tract. In this regard, an *in vitro* assay was performed to determine if the viability of *L. plantarum K16* and the stability of GABA was negatively affected by the conditions of the gastrointestinal tract.

The *in vitro* assay was conducted by preparing gastric and intestinal solutions trying to simulate gastrointestinal tract conditions. The gastric conditions were simulated by preparing a solution of 0.9% of sodium chloride (Scharlab) with 3 g/L of pepsin (Sigma). Then, three aliquots of this solution were isolated in sterile tubes and adjusted to pH 2, 4 and 6, respectively, as the pH value changes through the gastric tract. The intestinal solution was prepared with 3 g/L of porcine bile extract (Sigma), 6.5 g/L sodium chloride (Scharlab), 0.84 g/L potassium chloride (Scharlab), 0.22 g/L calcium chloride (Scharlab) and 1.39 g/L sodium hydrogen carbonate (Scharlab), and the initial pH was adjusted to 7.5.

The survival rate of *L. plantarum K16* was assessed by growing an overnight inoculum at 37 ºC in MRS broth and used to inoculate the experimental assay. Then, the survival rate of *L. plantarum K16* was evaluated under the gastric solutions at pH 2, 4 and 6, and the intestinal solution at pH 7.5, for 2 h at 37 ºC and agitation of 100 rpm. Samples were taken every 30 min to perform serial dilutions and plate the samples in MRS agar to measure the microbial growth (log CFU/mL).

The stability of GABA was measured also using the same type of gastric and intestinal solutions as described above. In this case, commercial GABA (Sigma) was weighted (300 mg) and added to sterile tubes to evaluate its stability in the gastric solutions at pH 2, 4 and 6, and in the intestinal solution at pH 7.5, for 2 h at 37 ºC and agitation of 100 rpm. Samples were taken every 30 min to quantify the concentration of GABA using UHPLC-MS.

3.6. Production of the microencapsulated functional ingredient

A protective microcapsule was designed to preserve the viability of *L. plantarum K16* and the stability of GABA in the gastrointestinal tract after ingestion. The

technological process used to develop these microcapsules was submitted as proposed patent named "Microcapsules containing gamma aminobutyric acid (Ref. EP21382550.8-Annex III)". For evident reasons, this subsection briefly explain the encapsulation process.

Before encapsulation, *L. plantarum K16* biomass and GABA were produced using tomato by-product as fermentation medium applying the best conditions found in the optimisation process described in the previous subsection 3.4. The tomato by-product substrate was enriched with 25 g/L of glucose, 12 g/L of yeast extract and 100% of minerals. The initial pH was adjusted to 5.5 and, after the sterilisation process, 500 mM of MSG were added to the medium. Then, the fermentation medium was inoculated with 1.2% of *L. plantarum K16* and fermented for 96 h at 34 ºC without shaking. Afterwards, the fermented tomato by-product containing GABA was sieved through a metallic mesh of 45 µm to remove the remaining tomato by-product. The fermented product was clarified by centrifuging for 15 min at 4,000 rpm and microfiltered through a polyethersulfone membrane of 0.22 µm pore size.

On the other hand, to get the highest amount of biomass of *L. plantarum K16* in the microcapsule, the probiotic was grown in tomato by-product using the optimised conditions for microbial cell growth (25 g/L of glucose, 12 g/L yeast extract and 100% minerals) for 24 h at 34 ºC. After this time, the microbial biomass was recovered by sieving the fermented medium through a metallic mesh of 45 µm pore size to remove the remaining tomato by-product. After that, the fermentation medium was centrifuged for 15 min at 4,000 rpm, the supernatant was discarded, and the biomass was washed with distilled water and further centrifuged for 15 min at 12,000 rpm to remove all the remaining water.

Afterwards, the encapsulation mixture was prepared with 1% of the recovered biomass of *L. plantarum K 16,* which was mixed with the clarified fermented tomato by-product enriched with GABA. In the next step, 2% of alginate (IMCD, Barcelona, Spain), used as encapsulation biopolymer, was mixed with the clarified fermented tomato by-product containing GABA and *L. plantarum K16.*

The encapsulation process was performed using an INOTECH vibrating-jet extrusion encapsulator (BUCHI, Barcelona, Spain). Figure 11 shows the different parts of the encapsulator equipment. The process started by introducing the encapsulation

mixture into the sterilised glass bottle. Then, a pressure of 450 mbares was applied to enhance the movement of the encapsulation mixture through the tube to arrive at the dripping system. Next, the dripping system was subjected to a frequency of 1,500 Hz producing the vibration of the extrusion nozzle with a diameter of 200 µm. The nozzle vibration made the jet cut, creating microspheres and ending up in a stirred gelation bath of calcium chloride 0.1M that hardened the envelope resulting in the microcapsules.

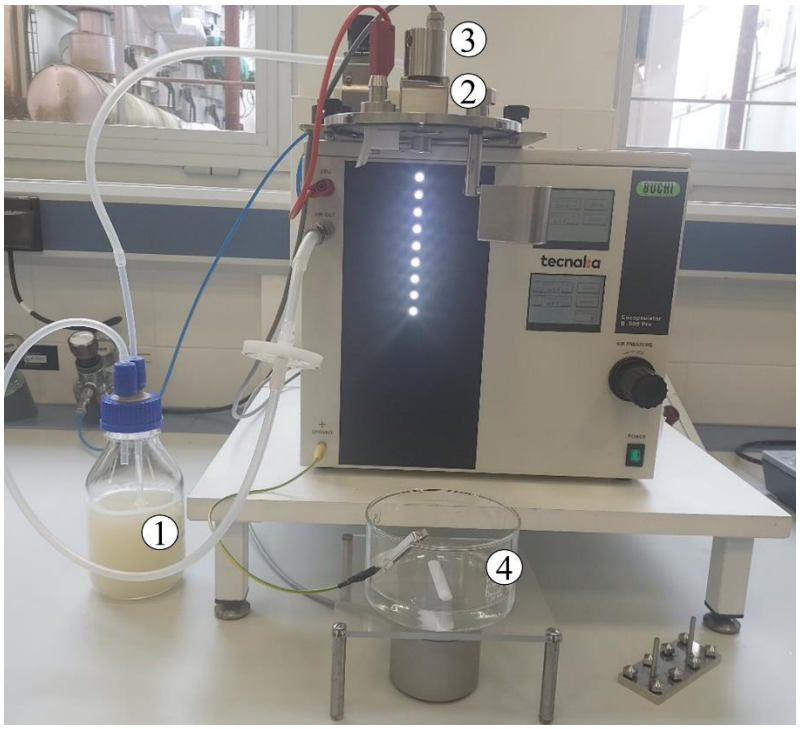


Figure 11: Vibrating-jet extrusion encapsulator: (1) sterilised glass bottle with encapsulation mixture; (2) dripping system; (3) frequency creator; (4) stirred gelation bath.

The microcapsules were sieved and rinsed with distilled water to remove the remaining calcium chloride. Finally, the microcapsules were immersed into sterilised milk to preserve the viability of the microorganisms in the drying step. After 30 min, the microcapsules were newly sieved and dried using a LyoBeta freeze dryer (Telstar, Madrid, Spain). A sample of microcapsules was taken to observe them in the microscope (Zeiss, Jena, Germany). The ELIX software (Zeiss) was used to determine the sphericity of the microcapsule and the size dispersion. After the drying process, the concentration of *L. plantarum K16* was assessed and the amount of GABA was measured. In this case, to perform the quantification of microorganisms, 100 mg of microcapsules were broken with sodium citrate (0.1 M) during 10 min stirring, and the microorganisms were grown

in MRS agar. Likewise, the broken capsules were used to determine the GABA concentration using UHPLC-MS.

3.7. Statistical analysis

The statistical analysis is detailed in the research articles included in Annexes I.II and I.III. IBM-SPSS statistics software version 25.0 (IBM, New York USA) was used for statistical analysis. One-way analysis of variance (ANOVA) was applied to determine the presence or absence of statistically significant differences between experiments. Bonferroni's method was used for pairwise comparisons. ANOVA was used to evalute the results obtained from the experiments described in sections 3.3, 3.4 and 3.5. In addition, Pearson and Rho Spearman analyses were used to calculate coefficients of correlation between different variables. Correlations were calculated for the results obtained from the experiments described in section 3.3.2. Statistical significance was declared at $P \leq 0.05$.

4. RESULTS AND DISCUSSION

The design of personalised therapies to address current high-prevalence diseases has gained importance over the past decades. As a result, developing new functional ingredients composed of probiotic microorganisms has received increased attention to satisfy this market demand. In this regard,, extensive research has been carried out to find more and better probiotics that could address a wide variety of beneficial effects on human health. This PhD thesis has developed a new functional ingredient characterised by the novelty of combining a new *L. plantarum K16* strain, isolated from the fermented food Kimchi, with the postbiotic metabolite GABA produced by a fermentation process using agri-food by-products as substrate media. According to the scientific literature, this functional ingredient intends to exert a beneficial effect on human health by acting in the intestine, guaranteeing a synergistic effect of the probiotic and GABA. Different agri-food by-products have been selected to be used as fermentation media due, on one hand, to their high environmental impact and, on the other hand, to their nutritional value for developing low-cost culture media. The most relevant results obtained for the development of the new functional ingredient are shown below and described progressively.

4.1. Literature review

An extensive literature review was performed to identify the most interesting microorganism for producing GABA that should be isolated, and the potential of this postbiotic metabolite was discussed in the review article included in Annex I.I. In this review article, the importance of the postbiotic metabolite GABA was argued coupled with the wide variety of its beneficial effects on human health against cardiovascular diseases, nervous systems disorders, diabetes, cancer, or asthma. Furthermore, the biosynthetic pathways used by microorganisms to produce GABA were explained in detail, paying more attention to *Lactobacillus spp.* strains and the most important fermentation parameters involved in GABA synthesis. Therefore, the review article was focused on using *Lactobacillus spp.* strains as GABA producers due to the well-known GAD machinery presented in *Lactobacillus spp*. As well this species has also been classified as one of the safest bacteria used as probiotics, showing a wide variety of beneficial health effects.

4.2. Isolation, identification and selection of *Lactiplantibacillus plantarum K16*

The fermented food kimchi, which is prepared using autochthonous and natural raw materials, showed a great community of LAB mainly composed of *S. thermophilus*, *L. plantarum* or *Lactococcus lactis.* However, only the strain *L. plantarum K16* presented the ability to produce GABA. Then, the growth kinetics of *L. plantarum K16* was assessed to ensure a high microbial cell growth and that the biomass produced was enough to carry out a biotechnological process facilitating the recovery and reducing the production expenses at an industrial level (Sabater et al., 2020). This growth kinetics of *L. plantarum K16* was assessed in MRS broth for 72 h to determine the highest microbial cell growth point. A concentration of 7.42 ± 0.02 log CFU/mL was inoculated, which significantly ($P \leq 0.05$) increased the biomass to 9.11 ± 0.09 log CFU/mL after 24 h of fermentation, and the microbial viability started to decrease from that time until 72 h (Figure 12). The pH of the fermentation media was dramatically reduced simultaneously as the microbial cell growth increased, and the concentration of glucose was wholly consumed after 24 h of fermentaion. In this case, *L.plantarum K16* presented a specific grow rate of 0.163 h^{-1} and a biomass yield of 0.096 grams of biomass produced *per* gram of substrate consumed, resulting in 9.01 log CFU/g.

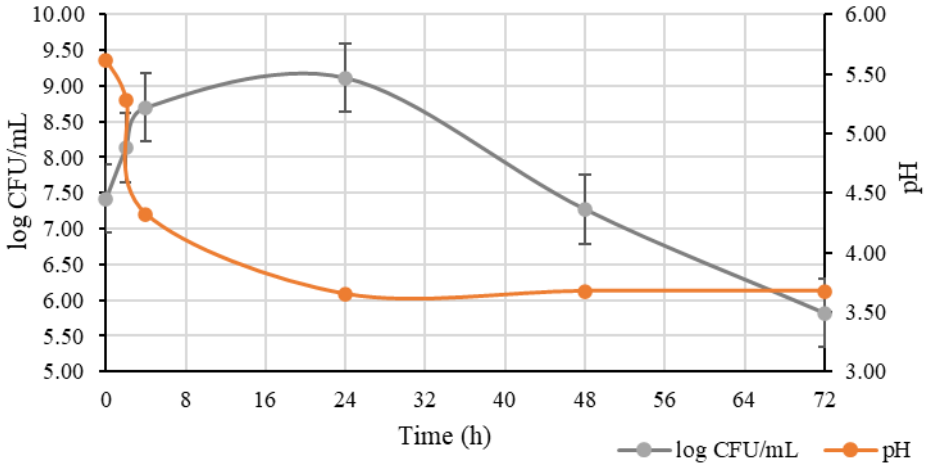


Figure 12: Microbial cell growth of *L. plantarum K16* strain (log CFU/mL) and the evolution of the medium pH for 72 h of fermentation in MRS broth.

The ability of *L. plantarum K16* to produce the postbiotic metabolite GABA and the great cell growth and biomass yield of this microorganism in the commercial MRS broth indicated that it was a suitable strain for developing the new functional ingredient.

4.3. Safety and probiotic effect of *Lactiplantibacillus plantarum K16*

Although *L. plantarum* strains are considered GRAS microorganisms and the EFSA conferred the Qualified Presumption of Safety status to *Lactobacillus* species (Liu et al., 2020; Ruiz Sella et al., 2021), it is necessary to study the probiotic capacity to ensure the safety and the probiotic potential of each strain. The procedure and the results obtained in the characterisation of the safety and the probiotic potential of *L. plantarum K16* strain are mainly presented in the research article included in Annex I.II. The following items were considered for the safety and probiotic characterisation of *L. plantarum K16*:

1. Haemolytic activity.
2. Resistance against antibiotics.
3. Carbohydrates metabolism.
4. Detection of enzymes with promoting health effect.
5. Antimicrobial effect against common pathogens.

The results showed that *L. plantarum K16* did not present haemolytic activity. As Figure 13a depicts, *L. plantarum K16* did not present the ability to break the red blood cells from sheep. Therefore, the growth of *L. plantarun K16* in Columbia blood agar supplied with 5% of sheep blood did not create a halo produced by the breakdown of the red blood cells, as observed in Figure 13b where a control bacteria showed beta-haemolysis.

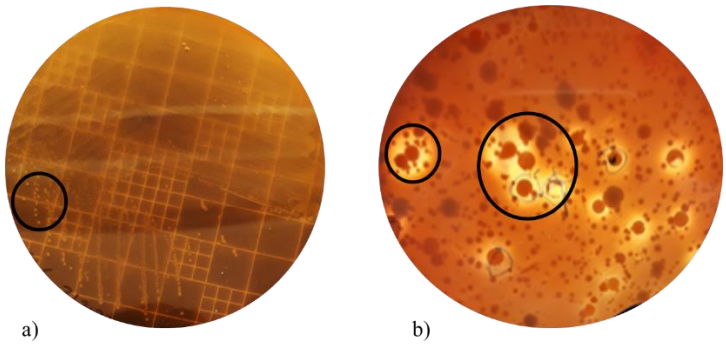


Figure 13: Image of Columbia blood agar plates with 5% of sheep blood: a) *L. plantarum K16* without haemolytic activity; b) Beta-hemolysis produced by control bacteria.

The results obtained for resistance, intermediate resistance and sensibility against antibiotics of *L. plantarum K16* strain are summarized in Figure 14. In the research article included in Annex I.II the concentration of each antibiotic tested and the inhibition halos observed are shown. *L. plantarum K16* presented resistance against some antibiotics that

could be useful to maintain the structure of the gut microbiota under antibiotic therapy (Machado et al., 2022). The disk-diffusion method to test antibiotic resistance carried out in this investigation gives reliable qualitative information. However, it should be emphasized that EFSA indicates that before the commercialisation of the final product, it is necessary to determine the minimum inhibitory concentration, and genetically identify if the resistance genes are transferable (EFSA, 2012).

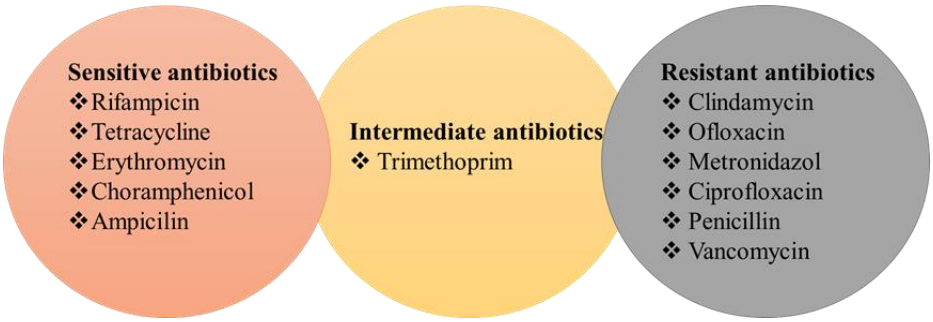


Figure 14: Summary of the resistance, intermediate resistance and sensibility of *L. plantarum K16* strain against different antibiotics using the disk diffusion method.

Likewise, API strips were used to evaluate the metabolism of carbohydrates and the activity of other enzymes that could have a beneficial effect on human health. Figure 15 lists the carbohydrates that *L. plantarumm K16* can metabolize and the research article included in Annex I.II shows all the carbohydrates tested. For instance, the results showed that *L. plantarum K16* could metabolize several monosaccharides such as glucose, galactose, or fructose, that could stimulate microbial cell growth because they can be easily used as energy sources (Hedberg et al., 2008). Furthermore, *L. plantarum K16* also metabolized several disaccharides and glucosides (Figure 15) where it is essential to highlight the ability of this microorganism to use amygdaline, which is not always found in all *L. plantarum* strains. In consequence, the metabolization of amygdaline could be considered an attractive probiotic characteristic as this sugar can present a cytotoxic effect and produce the degeneration of nerves (Gebreselassie et al., 2016). *L. plantarum K16* also metabolized polyols and raffinose (Figure 15). Using raffinose could increase the absorption of essential nutrients, stimulate the digestion process, help preserve the gut microbiota structure and enhance the production of organic acids (Mao et al., 2018; Xiao et al., 2015). *L. plantarum K16* also degraded the polysaccharide inulin, increasing the production of SCFA such as butyric acid, which could help to maintain the microbiota and prevent the development of gastrointestinal disorders in humans (Shoaib et al., 2016).

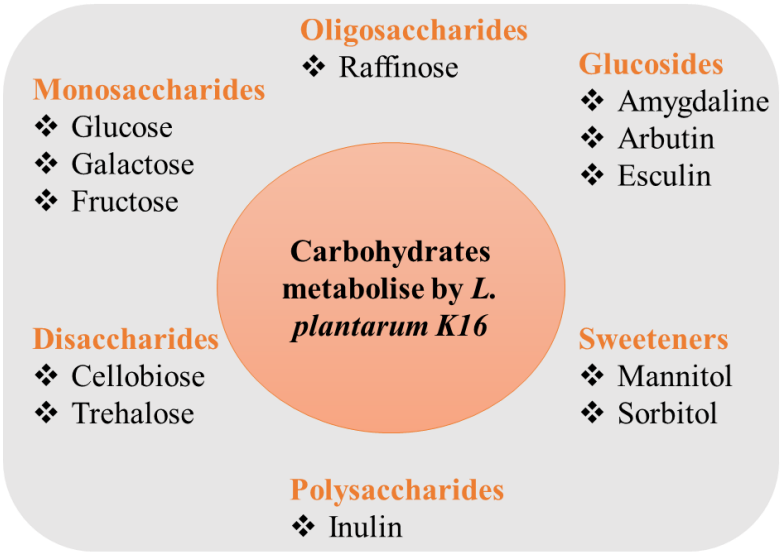


Figure 15: Summary of the carbohydrates (monosaccharides, disaccharides, oligosaccharides, glucosides, sweeteners and polysaccharides) that *L. plantarum K16* strain can metabolize.

L. plantarum K16 could also play a vital role in the metabolism of carbohydrates, lipids, or proteins, enhancing the digestion and metabolism of humans using several enzymes. The research article included in Annex I.II presents different enzymes with a healthy effect which were tested for *L. plantarum K16*, and the results showed positive activity for these enzymes. In this regard, *L. plantarum K16* showed a slight lipase activity that could enhance lipoprotein metabolism by its ability to degrade fat. Likewise, the activity of naphthol-AS-BI-phosphohydrolase and valine, cystine and leucine arylamidases also enhances the absorption of nutrients and stimulate digestion (Oberg et al., 2016). Furthermore, the reported high activity of β-galactosidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase could further improve the degradation of carbon sources. In particular, a good activity of β-galactosidase could reduce lactose intolerance by degrading this sugar. On the other hand, the N-acetyl-β-glucosaminidase could help avoid the colonisation of *Aspergillus niger*, as this enzyme could break down the cell wall chitin of this pathogen (Colombo et al., 2018).

The antimicrobial effect of *L. plantarum K16* strain was further evaluated against the common foodborne pathogens such *E. coli*, *S. typhimurium* and *L. monocytogenes.* The research article included in Annex I.II presents the inhibition halos obtained using the disk-diffusion method and the agar well-diffusion test. These results indicated that *L. plantarum K16* had an inhibitory effect against the Gram-negative bacilli *E. coli* and *S. typhimurium.* However, *L. plantarum K16* did not present an inhibitory effect against *L.*

monocytogenes. Therefore, the antimicrobial effect of *L. plantarum K16* was further evaluated through *in vitro* studies using Caco2 cell culture against the same pathogens (*E. coli, S. typhimurium* and *L. monocytogenes*). In this case, a protective, competitive and displacement assays were conducted using *L. plantarum K16* and a pathogen in one-to-one experiment. First, the adhesion capacity of *L. plantarum K16* to adhere to Caco2 cell layer was evaluted, showing an adhesion percentage of 67.25%. As illustrates in Figure 16 , the protective effect of *L. plantarum K16* against these pathogens was performed by first adding *L. plantarum K16* to allow this bacteria to attach to the Caco2 cell layer. Then, the pathogens were added to determine if *L. plantarum K16* prevented these pathogens from binding. The final results showed that the probiotic could not reduce the attachment of *E. coli* and *L. monocytogenes.* However, as Figure 17 shows, *L. plantarum K16* had a protective effect by significantly reducing ($P \leq 0.05$) the adhesion of *S. typhimurium* by 25% compared to the ability of this pathogen to adhere alone.

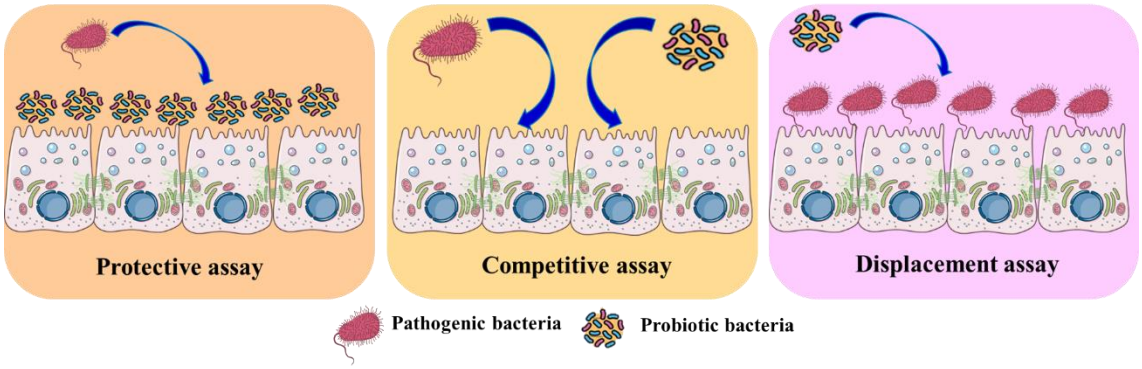


Figure 16: Scheme of the protective, competitive and displacement assays carried out in Caco2 cells using the probiotic bacteria (*L. plantarum K16)* and the pathogenic bacteria (*E. coli, S. typhimurium* and *L. monocytogenes).*

In the competitive study *L. plantarum K16* and the pathogens where added at the same time. This assay evaluated if *L. plantarum K16* was able to prevent the adhesion of pathogens by occupaying niches. In this case, the results showed that the adhesion of *E. coli* and *L. monocytogenes* was not reduced in the presence of *L. plantarum K16* (Figure 17). Nevertheless, the competitive study between *S. typhimurium* and *L. plantarum K16*, the adherence of *S. typhimurium* decreased by almost 17% ($P \leq 0.05$) in comparison to the adherence of *S. typhimurium* without the presence of *L. plantarum K16.*

As Figure 16 depicts, the displacement effect of *L. plantarum K16* against these attached pathogens was performed by first adding the pathogens to allow the probiotic

bacteria to attach to the Caco2 cell layer. Then, *L. plantarum K16* was added to determine if it could detach the attached pathogens. No significant decrease ($P > 0.05$) of *S. typhimurium, L. monocytogenes* or *E. coli* adhesion was observed during the displacement study (Figure 17). According to these results, *L. platarum K16* showed more inhibitory potential against *S. typhimurium.* An important inhibitory effect of *L. plantarum* against *S. typhimurium* has been reported in a greater number of studies than in others. For example, Jamyuang et al. (2019) reported that *Lactobacillus* strains had a robust protective effect against the adhesion of *S. typhimurium* by reducing from 30 to 40% the pathogen, which was much better than the anti-adhesive effect observed against *E. coli.* Zawistowska-Rojek et al. (2022) reported that *Lactobacillus spp.* also reduced the adherence of pathogenic bacteria. Still, in this case, the anti-adhesive effect was more important against *E. coli* than *S. typhimurium.*

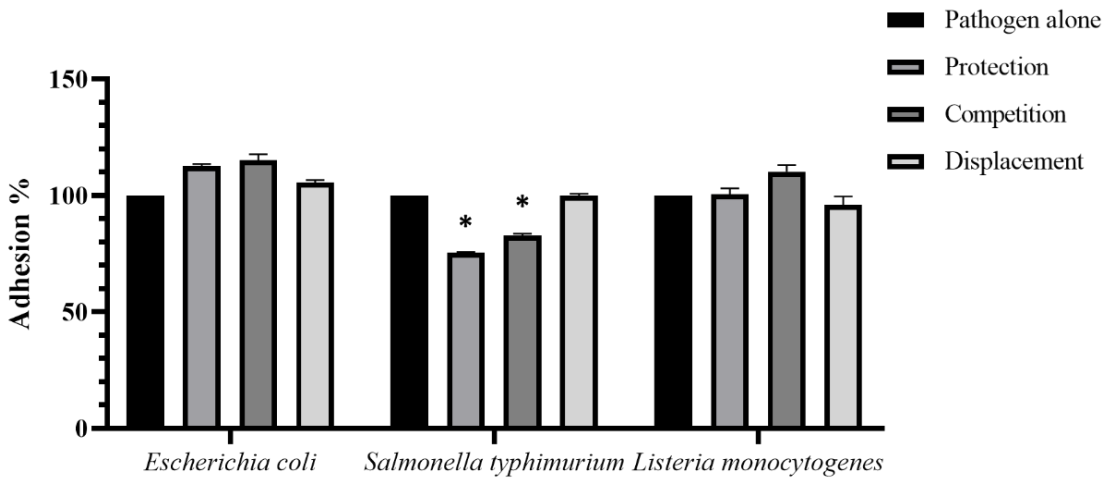


Figure 17: Percentage of adhesion of *E. coli, S. typhimurium and L. monocytogenes* without and with *L. plantarum K16*; * $P \leq 0.05$).

In short, the results of the caracterization of the safety and probiotic effect of *L. plantarum K16* strain indicated that this microorganism has the potential to stimulate digestion and absorption of nutrients to promote health, and at the same time, *L. plantarum K16* showed a potential inhibitory effect against pathogens such as *S. typhimurium*. The book chapter entitled "The role of probiotics in nutritional health: probiotics as nutribiotics" (Chavarri et al., 2022), included in Annex II.I as supplementary material, extensively discussed the nutritional health benefit of the probiotics such as *L. plantarum* strains.

4.4. Production of gamma-aminobutyric acid by *Lactiplantibacillus plantarum K16*

The synthesis of postbiotic metabolites is close related to the composition of the culture media and the cultivation parameters. The book chapter entitled "Secondary Metabolites From Probiotic Metabolism" (Chávarri et al., 2021), included as supplementary material in Annex II.II, discusses how several fermentation parameters and substrate composition can influence the synthesis of different postbiotic metabolites. An extensive literature review was performed to determine the main parameters that could strongly impact the fermentation process to synthesize the postbiotic metabolite GABA. The review article included in Annex I.I and the research article in Annex I.II reported how environmental factors, such as temperature, pH, and medium supplements (carbon and nitrogen sources, or MSG), and the cultivation time could modulate the activation of the GAD pathway and, thus, influence GABA yield. After identifying the main factors influencing GABA, an experiment was designed to individually study the effect of each parameter on the growth of *L. plantarum K16* and to optimize the fermentation conditions in order to produce the highest concentration of GABA. In this first step, a commercial medium was used to control all the nutrients that may affect the yield of GABA. Then, tomato, orange, green pepper and apple by-products were used to prepare fermentation media and select the most suitable to develop the functional ingredient enriched with the greatest concentration of GABA.

4.4.1 Postbiotic production using commercial broth

As previously described in Material and Methods subsection, MRS broth was used to study the impact of incubation temperature, yeast extract concentration, fermentation time, percentage of inoculum, initial pH, MSG and glucose concentrations on the production of GABA by *L. plantarum K16*. The initial fermentation conditions were set to 17 g/L of yeast extract, 5 g/L of glucose, 500 mM of MSG, an inoculum of 1%, initial pH of 5.5 and an incubation temperature of 30 ºC. Focusing on fermentation time, previous studies reported that the best time to produce the highest GABA amount could be 24 (Sahab et al., 2020), 48 h (Shan et al., 2015) or even 72 h (Zhang et al., 2017). Therefore, to ensure the best time for *L. plantarum K16* to produce the greatest concentration of GABA, a timeline analysis was performed by sampling every 24 h during a fermentation period up to 72 h. Under initial fermentation conditions, *L. plantarum K16* significantly ($P \leq 0.05$) increased the GABA production reaching the

highest highest amount after 72 h (421.96 ± 43.12 mg/L; Figure 18) together with a cell growth of 9.13 ± 0.06 log CFU/mL and a pH of 4.44 ± 0.02 (Figure 19). In consequence, 72 h was establish as the best fermentation time for subsequent OFAT experimental trials.

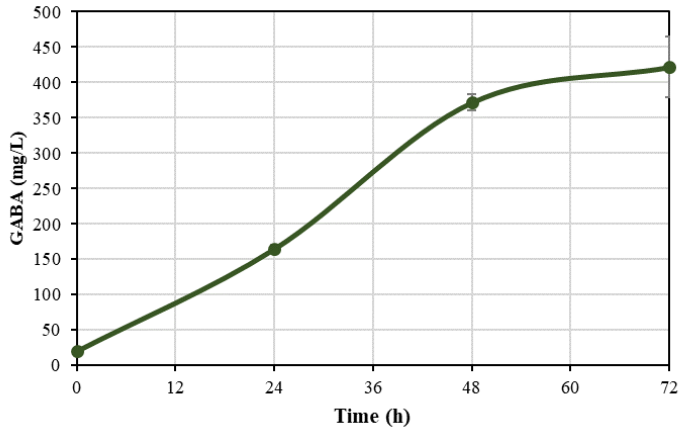


Figure 18: Evolution of gamma-aminobutyric acid (GABA) production (mg/L) using *L. plantarum K16* under initial fermentation conditions (17 g/L yeast extract, 30 ºC incubation temperature, 72 h fermentatio time, 1% of inoculum, initial pH 5.5, 500 mM monosodium glutamate and 5 g/L of glucose).

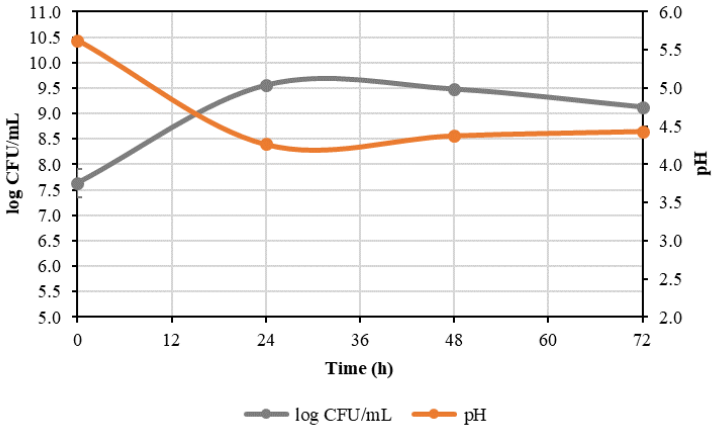


Figure 19: Evolution of microbial cell growth (log CFU/mL) and pH media using *L. plantarum K16* under initial fermentation conditions (17 g/L yeast extract, 30 ºC incubation temperature, 72 h fermentatio time, 1% of inoculum, initial pH 5.5, 500 mM monosodium glutamate and 5 g/L of glucose).

The results of the experimental trials using MRS broth are presented in the research articles included in Annex I.II (incubation temperature, yeast extract concentration, and fermentation time) and Annex I.III (percentage of inoculum, initial pH, and concentrations of MSG and glucose). In both research articles, the effect of each

parameter was deeply explained indicating how this condition may influence the synthesis of GABA by *L. plantarum K16* strain and comparing the results with other studies. Also, both research articles show tables containing the results for GABA concentration, medium pH and microbial cell growth obtained from the OFAT experimental design. As mentioned above in Materials and Methods subsection, Figure 9 depicts a scheme of the parameters evaluated in each step of the experimental design.

The experimental design started by optimising the fermentation temperature to reach the thermodynamic equilibrium of the GAD biosynthetic pathway. As Figure 20a shows, the increase in the incubation temperature from 30 ºC, fixed as initial fermentation condition, to 34 ºC significantly rised ($P \leq 0.05$) the biosynthesis of GABA producing 561.36 ± 28.26 mg/L. This temperature significantly enhanced ($P \leq 0.05$) the GABA yield by 33%, but the microbial cell growth significantly decreased ($P \leq 0.05$) to 7.44 ± 0.06 log CFU/mL (Figure 24).

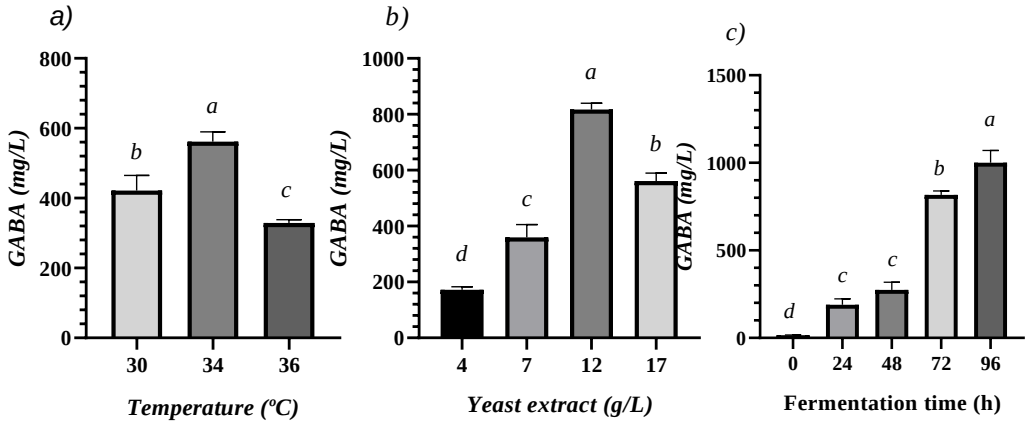


Figure 20: Gamma-aminobutyric acid (GABA) production by *L. plantarum K16* in MRS broth under different: a) Temperature; b) Yeast extract concentration; c) Fermentation time.

Then, different concentrations of yeast extract were used to determine how the nitrogen source could modify the GABA production. Figure 20b shows that GABA synthesis increased at the same time as the concentration of yeast extract was higher, reaching the greatest concentration with 12 g/L of yeast extract. However, when the concentration of yeast extract increased up to 17 g/L the concentration of GABA was significantly lower ($P \leq 0.05$) to that obtained using 12 g/L. At 12 g/L of yeast extract concentration, 816.84 ± 22.44 mg/L of GABA, with a microbial cell growth of 7.94 ± 0.06 log CFU/mL, and pH of 4.42 were obtained. Hence, 12 g/L yeast extract which

increased the yield of the process by 45.5% was selected to continue the OFAT experimental trials (Figure 24).

In addition, a new timeline was performed, expanding the fermentation time to 96 h to determine whether GABA amount increased over this time, or the production was either reduced or not increased. The results showed that the GABA production significantly increased ($P \leq 0.05$) after 96 h of fermentation yielding 1000.23 ± 70.82 mg/L of GABA, microbial cell growth of 6.99 ± 0.03 log CFU/mL, and pH of 4.42 (Figure 20c). Figure 24 shows that increasing the fermentation time from 72 h to 96 h the GABA yield raised by 22.5%.

OFAT experimental trials for the study of the inoculum percentage showed that the yield of GABA was 42% higher ($P \leq 0.05$) (Figure 24) when the inoculum percentage increased from 1% (7.44 ± 0.06 log CFU/mL) to 1.2% (7.5 ± 0.03 log CFU/mL). With a 1.2% of inoculum the microbial cell growth was 7.31 ± 0.14 log CFU/mL and GABA amount 1419.93 ± 57.47 mg/L (Figure 21a).

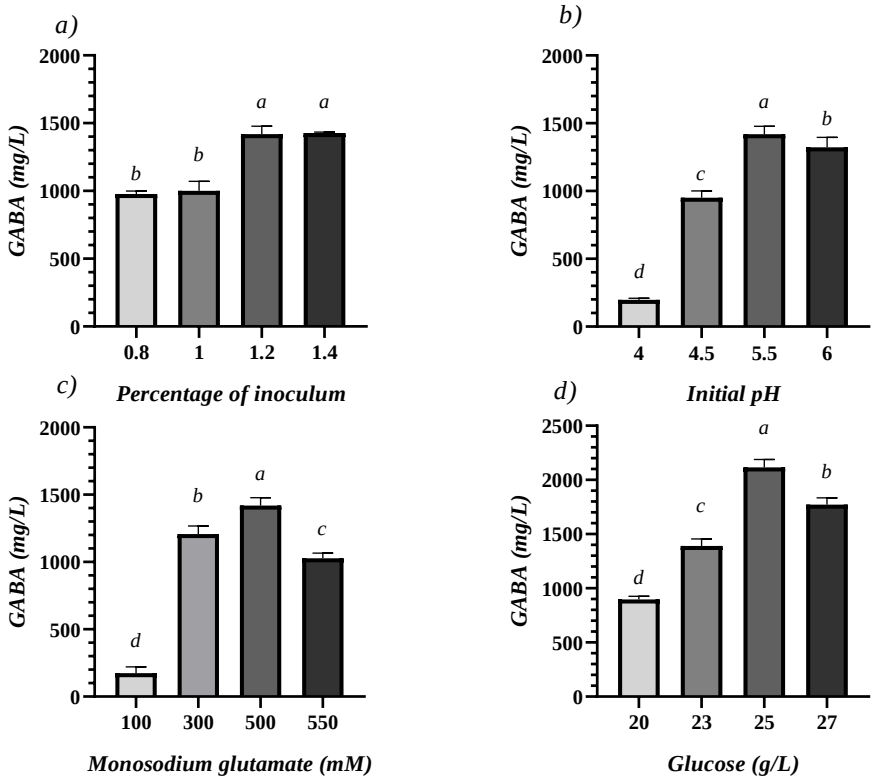


Figure 21: Gamma-aminobutyric acid (GABA) production by *L. plantarum K16* in MRS broth under different: a) Percentage of inoculum (%); b) Initial pH; c) Monosodium glutamate; d) Glucose.

Regarding the initial pH and MSG concentration, the results of the OFAT experimental trials indicated that the highest concentration of GABA was reached ($P \leq 0.05$) using an initial pH of 5.5 (Figure 21b) and when MSG concentration was 500 mM (Figure 21c). Finally, the results corresponding to the effect of the concentration of glucose indicated that the GABA yield was increased ($P \leq 0.05$) up to 49% when the concentration of glucose was 25 g/L (Figure 24).With this glucose concentration 2115.70± 73.83 mg/L of GABA, a microbial cell growth of 7.4 ± 0.14 log CFU/mL, and a pH of 4.43 were obtained (Figure 21d).

Taking into account the results described above, the optimal fermentation conditions were set to 12 g/L of yeast extract, 25 g/L of glucose, 500 mM of MSG, an inoculum of 1.2%, initial pH of 5.5, incubation temperature of 34 ºC and 96 h of fermentation time. Furthermore, a new timeline study expanding the fermentation time to 120 h was performed applying the optimal fermentation conditions to determine whether GABA amount increased over this time, or the production was either reduced or not increased. The microbial cell growth, medium pH and GABA concentration were measured. As Figure 22 depicts, the microbial cell growth exponentially grew after 24 h of fermentation reaching a value of 9.5 ± 0.02 log CFU/mL.

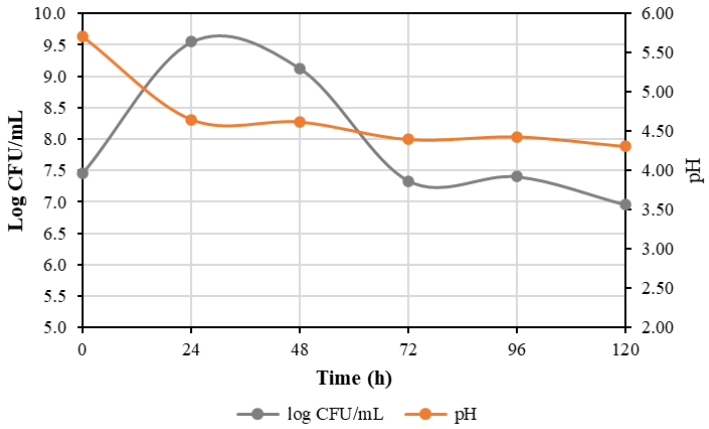


Figure 22: Evolution of microbial cell growth (log CFU/mL) and medium pH using *L. plantarum K16* under optimal fermentation conditions (12 g/L yeast extract, 34 ºC incubation temperature, 96 h fermentatio time, 1.2% of inoculum, initial pH 5.5, 500mM monosodium glutamate and 25 g/L of glucose) in MRS broth.

Afterwards, the microbial growth significantly decreased ($P \leq 0.05$) simultaneously with the increase in the production of GABA, which progressively raised

($P \leq 0.05$) during the fermentation timeline until it reached a maximum at 96 h (Figure 23).

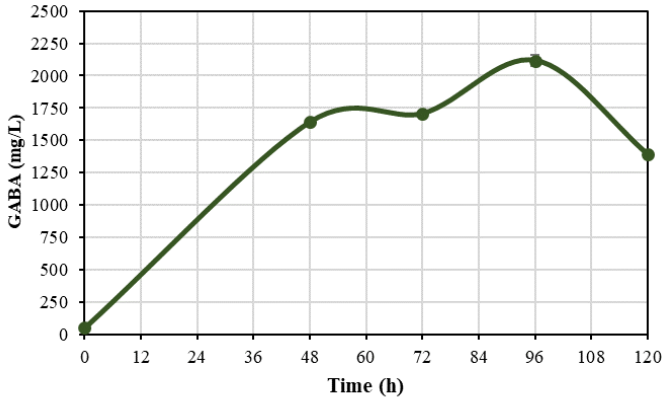


Figure 23: Evolution of gamma.aminobutyric acid (GABA) production using *L. plantarum K16* under optimal fermentation conditions (12 g/L yeast extract, 34 ºC incubation temperature, 96 h fermentatio time, 1.2% of inoculum, initial pH 5.5, 500mM monosodium glutamate and 25 g/L of glucose) in MRS broth.

A comparison between the initial and the optimal fermentation conditions (Figure 24) showed that using the optimal conditions significantly increased ($P \leq 0.05$) the GABA yield by 401.4% producing 2115.70 ± 73.83 mg/L of GABA, compared to the 421.96 ± 43.12 mg/L of GABA obtained with the initial fermentation conditions. With the initial fermentation conditions the microbial cell growth was maintained over time after reaching the highest concentration. On the contrary, using the optimal conditions, after getting the highest microbial cell growth, a severe reduction of log CFU/mL was observed over time coupled with an increase in GABA production. These results suggested that the initial fermentation conditions enhanced more the cell duplication and the cell density maintenance of *L. plantarum K16* than the optimal ones. On the other hand, the metabolism of *L. plantarum K16* looked more focused on the GABA production than cell duplication using the optimal fermentation conditions.

As a result, the OFAT optimisation process carried out could be considered highly effective increasing the postbiotic metabolite GABA compared to other optimisation studies (Harnentis et al., 2019; Tajabadi et al., 2015; Zareian et al., 2013).

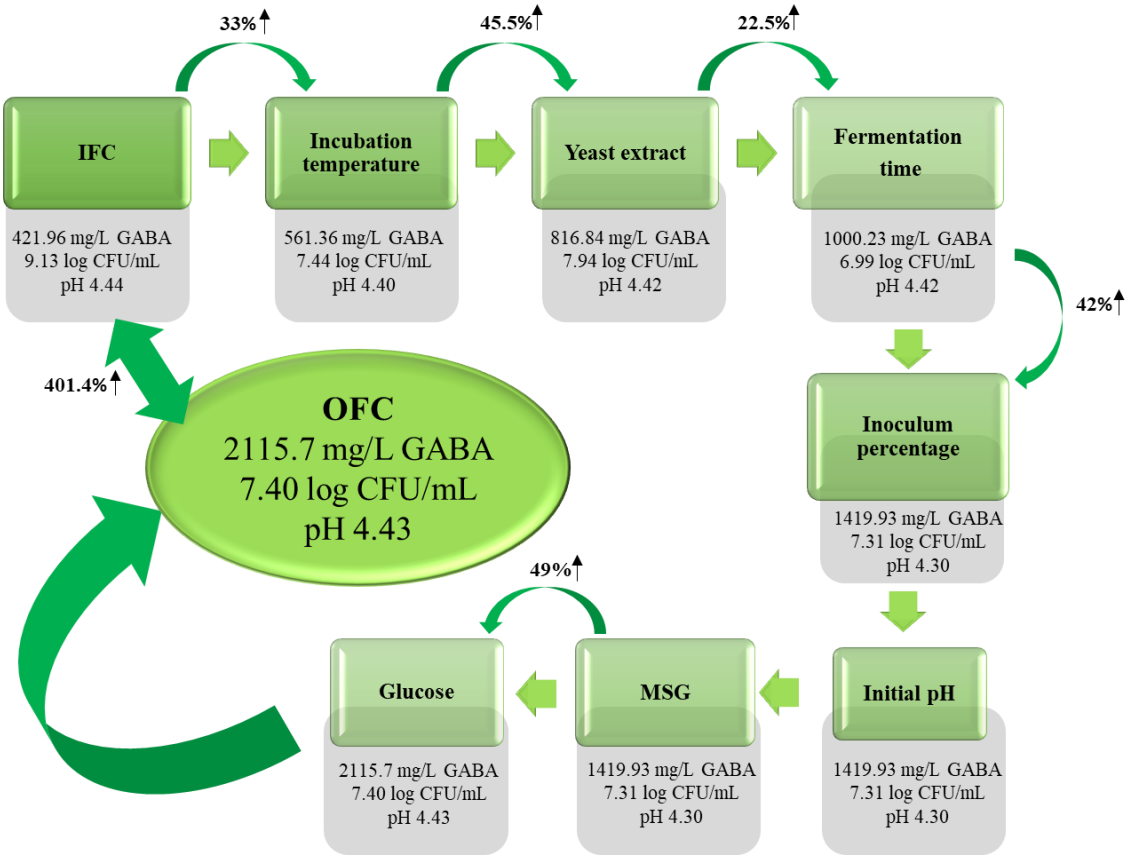


Figure 24: Scheme of the one-factor-at-a-time experimental design showing the best results of gamma-aminobutyric acid (GABA) obtained in each step and highlighting how these parameters enhanced the synthesis of GABA expressed as percentages. IFC, initial fermentation conditions; OFC, optimal fermentation conditions; MSG, monosodium glutamate.

4.4.2 Postbiotic production using agri-food by-products

Despite the high yield of GABA obtained by *L. plantarum K16* strain using MRS broth, this commercial medium is not recommended for scale-up production since it contains a high concentration of nutrients, which considerably can increase the production process cost (Zhang et al., 2020). Therefore, using agri-food by-products as fermentation substrates could be an excellent alternative to enhance microbial growth and produce GABA. Currently, these by-products are considered potential pollutants as they are usually burned or dumped in landfills, or they could be used for animal feeding. Hence, using agri-food by-products as fermentation media can be an excellent way to decrease the environmental impact, reduce the expenses of the biotechnological process and re-value sources of nutrients (Andreadis et al., 2022; Rangel et al., 2020).

This study used agri-food by-products of orange, apple, green pepper, and tomato to prepare culture media to produce GABA by applying the optimal fermentation

conditions previously observed using commercial MRS broth. The research article included in Annex I.III contains the results of the preliminary fermentation trials carried out with these by-products and their nutritional composition. The GABA yield using these four agri-food by-products was compared with the results obtained using commercial MRS broth (Figure 25). This fermentation trials helped to select the most suitable agri-food by-product to develop the final functional ingredient.

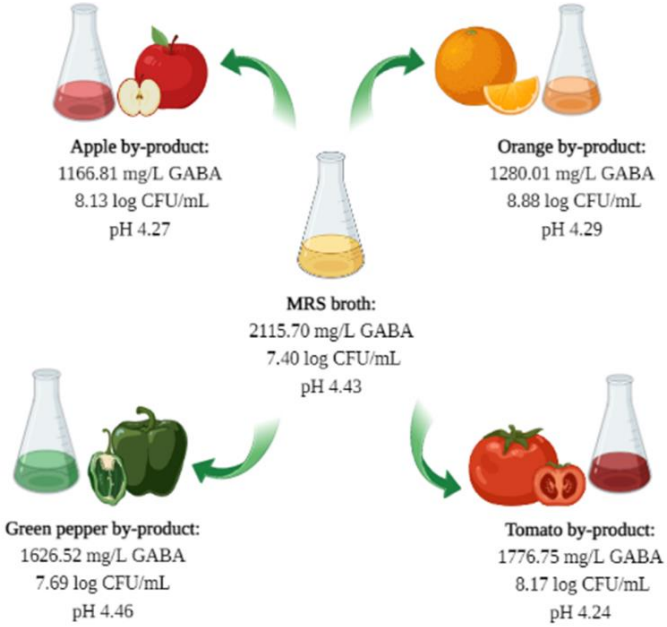


Figure 25: Gamma-aminobutyric (GABA) production, microbial cell growth and pH of *L. plantarum K16* strain using commercial MRS broth compared to apple, orange, green pepper and tomato by-products used as fermentation substrates. The fermentation conditions in all cases were the following: 12 g/L yeast extract, 34 ºC incubation temperature, 96 h fermentatio time, 1.2% of inoculum, initial pH 5.5, 500mM monosodium glutamate and 25 g/L of glucose.

The fermentation trials showed that the lowest GABA yield ($P \leq 0.05$) was observed using apple by-products, reaching a GABA concentration of 1166.81 ± 27.46 mg/L, with a microbial cell growth of 8.13 ± 0.04 log CFU/mL (Figure 25). These results slightly increased using orange by-products (1280.01 ± 59.22 mg/L of GABA and 8.88 ± 0.14 log CFU/mL). The amount of GABA produced was even significantly higher ($P \leq 0.05$) when green pepper (1626.52 ± 55.9 mg/L of GABA) and tomato (1776.75 ± 109.49 mg/L of GABA) by-products were used as fermentation substrates (Figure 25). Although all these agri-food by-products were successfully used to produce GABA, the yield was significantly lower ($P \leq 0.05$) than that achived in commercial MRS broth. The variability

in the GABA yield among agri-food by-products and MRS broth could be related to the different nutritional profiles.

On the other hand, these four agri-food by-products are a natural source of nutrients with variable composition, as it is reported in the research article included in Annex I.III. For this reason, glucose was added to the by-products to ensure that there was an accessible source of carbon for the microbial growth and, enough nitrogen source, yeast extract and MSG were also added, to favour GABA synthesis. Therefore, the correlation between the production of GABA and the concentration of protein, L-Glu and sugars of each agri-food by-product was made. The results showed that a high GABA yield was positively correlated with the protein content and the concentration of L-Glu in the agri-food by-product. However, a negative correlation with GABA yield was observed when the by-products presented higher concentration of carbohydrates.In this regard, a higher concentration of carbohydrates could play a more critical role in the metabolic pathways involved in cell duplication. According to the results on GABA yield obtained in the fermentation trials, the tomato by-product was selected as the fermentation substrate to develop the final functional ingredient. On the other hand, Lu et al.(2019) and Laranjeira et al. (2022) have reported that the sustainable valorisation of tomato pomace (by-product) through the development of new functional ingredients is a good source of beneficial health compounds such as amino acids, dietary fibre, unsaturated fatty acids, lycopene or phenolic compounds.

4.5. Development of a fermentation medium using tomato by-products

The next step in developing the functional ingredient was focused on evaluating the effect of the nutrient concentration on the microbial growth and GABA production by *L. plantarum K16.* Two experimental studies were performed independently to determine the best conditions to get the highest microbial cell growth and the greatest GABA yield using tomato by-product.

4.5.1 Microbial cell growth using tomato by-products

The microbial cell growth of *L. plantarum K16* strain using tomato by-product was statistically analysed to determine how different nutrients could interact and predict the highest microbial cell growth. Glucose, yeast extract and minerals were the nutrients selected to develop a Box-Behnken experimental design because they are considered essential elements for the growth of *L. plantarum* strains (Hayek & Ibrahim, 2013). The

supplementation of different concentrations of glucose (0, 12.5 and 25 g/L), yeast extract (0, 6 and 12 g/L) and minerals (0, 50 and 100%) was used to determine their effect on the cell growth of *L. plantarum K16* strain during 24 h of fermentation. The growth of *L. plantarum K16* observed in these experiments allowed the development of a predictive model to detect the optimal concentration of glucose, yeast extract and minerals to get the highest microbial cell growth. The following equationwas obtained by considering the parameters that mainly influence the growth of *L. plantarum K16*:

$$Y = 9.15+0.16A-0.043B+0.23C+0.24AB-0.21A^2$$

where *Y* isthe predicted microbial growth (log CFU/mL), *A* is the glucose concentration, *B* the yeast extract concentration, and *C* the percentage of minerals. This equation was used to calculate the predicted microbial growth and the absolute deviation observed between the experimental data and the predictive microbial cell growth to ensure its reliability (Table 4).

Table 4: Experimental and predicted microbial growth (log CFU/mL) of *L. plantarum K16* obtained from a Box-Behnken experimental design combining different concentrations of glucose (g/L), yeast extract (g/L) and minerals (%) using tomato by-product as fermentation medium. AD, absolute deviation between experimental and predicted microbial growth

Glucose concentration	***Yeast extract concentration***	***Minerals***	***Experimental microbial growth***	***Predicted microbial growth***	***AD***
0	0	50	9.05	9.06	0.01
12.5	6	50	9.20	9.15	0.05
12.5	12	0	8.81	8.87	0.06
25	0	50	9.05	8.90	0.15
0	6	100	9.02	8.78	0.24
25	6	0	8.79	8.87	0.08
12.5	0	0	8.83	8.99	0.16
12.5	6	50	9.26	9.15	0.11
12.5	6	50	9.23	9.15	0.08
12.5	0	100	9.20	9.42	0.22
12.5	12	100	9.50	9.34	0.16
0	12	50	8.26	8.50	0.24
0	6	0	8.78	8.55	0.23
25	12	50	9.22	9.30	0.08
25	6	100	9.34	9.33	0.01

The suitability of the predictive model was evaluated by looking at the determination coefficient (R^2) and the adjusted R^2, which were 0.80 and 0.69, respectively. Hence, an R^2 higher than 0.75 indicates good quality and accuracy of the model (Sharma et al., 2021a). The significant relationship between the independient variables and the response variable observed in the model was evaluated by ANOVA. Table 5 shows that the concentration of glucose and the percentage of minerals supplied to the fermentation media significantly affect ($P \leq 0.05$) the growth of *L. plantarum K16.* On the contray, the yeast extract concentration did not significantly influence ($P > 0.05$) the microbial cell growth.

Table 5: Results of the ANOVA for the independient variables included in the predictive model for the microbial growth of *L. plantarum K16* strain using tomato by product as fermentation medium.

Independient variables	***Sum of Squares***	**F-value**	***P***
A	0.21	7.12	0.026
B	0.01	0.49	0.500
C	0.43	14.65	0.004
AB	0.23	7.90	0.020
A^2	0.16	5.56	0.043

A=Glucose; *B*=Yeast extract ; *C*=Minerals

Therefore, the cultivation media needed a combination of minerals with at least one carbon source, mainly glucose, to enhance microbial cell growth (Kwoji et al., 2022). Nitrogen sources such as yeast extract are also essential as they can be used as amino acid, peptides, nucleic acids, minerals, vitamins and even carbon sources (Setya Utama et al., 2020). Furthermore, it has been reported that buffereing agents such as sodium acetate, ammonium citrate or dipotassium phosphate are required to control the medium pH while the bacteria is growing (Hayek et al., 2019). Likewise, Mousavi et al. (2011) indicated that glucose was the main energy and carbon source for *L. plantarum* strains over other sources, enhancing the growth rate. Wegkamp et al. (2010) highlighted the importance of minerals and glucose supplementation in the growth of *L. plantarum WCFS1* strain. Specifically, these authors identified magnesium as essential to enhance enzymatic reactions and manganese to protectagainst oxidative agents. Iino et al. (2002) suggested that sodium acetate could enhance the activation of the glycolytic pathway increasing the consumption of glucose, producing lactic acid and improving the growth

yield. Moreover, citrate addition could enhance the assimilation of glucose by *L. plantarum* strains (Savard & Champagne, 2017). In this regard, Yang et al. (2022) reported that *L. plantarum* strains could present a citrate-glucose co-metabolism where the fermentation of citrate is related to glycolisis acting as an important energy source producing proton motive force.

The relationship between different ranges of two of the independent variables studied and the third one fixed on its central point value was represented in three-dimensional response surface curves. The response surface matrix depicted inFigure 26 shows how the combination of glucose and minerals concentrations coupled with 6 g/L of yeast extract could modify the microbial cell growth of *L. plantarum K16*.

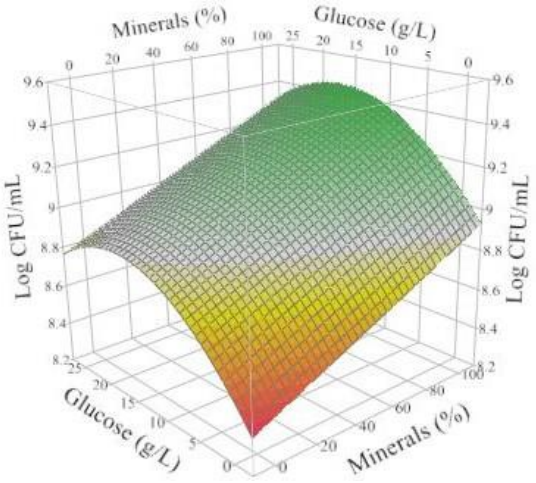


Figure 26: Response surface matrix representing the combined effect of glucose and minerals concentrations on the *L. plantarum K16* microbial growth using tomato by-product as fermentation medium. Yeast extract concentration of 6 g/L.

Consequently, the microbial growth significantly increased ($P \leq 0.05$) with the higher concentration of minerals and glucose. As Table 4 shows, when fermentation was carried out by adding 6 g/L of yeast extract without supplementation of minerals and glucose, the microbial growth was 8.78 log CFU/mL. However, with this yeast extract concentration, no glucose and 100% of minerals, the growth hit 9.02 log CFU/mL. Furthermore, supplying 25 g/L of glucose coupled with 100% of minerals and 6 g/L of yeast extract resulted in a microbial cell growth of 9.34 log CFU/mL.

Focusing on the relationship between the concentrations of yeast extract and glucose, using 50% of minerals, the response surface matrix indicated that the increase of glucose and yeast extract concentrations enhanced the growth of *L. plantarum K16*

(Figure 27). In this case, when no glucose or yeast extract was used the microbial growth was 9.05 log CFU/mL. However, 9.22 log CFU/mL was quantified when 25 g/L of glucose and 12 g/L of yeast extract together with 50% of minerals were added to fermentation medium (Table 4).

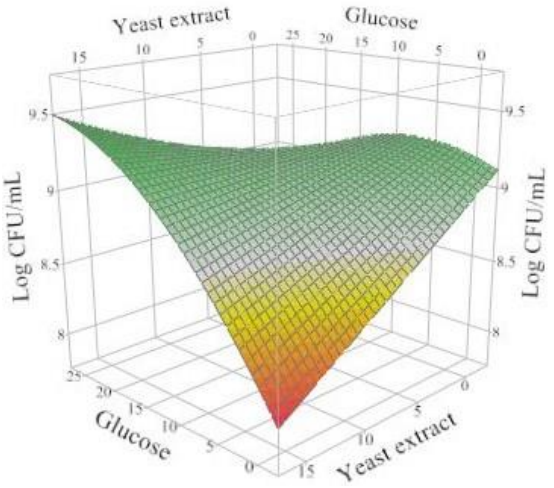


Figure 27: Response surface matrix representing the combined effect of glucose and yeast extract concentrations on the *L. plantarum K16* microbial growth using tomato by-product as fermentation medium. Minerals concentration of 50%.

Furthermore, the response surface matrix depicting the relationship between the concentrations of yeast extract and minerals, with the addition of 12.5 g/L of glucose, clearly highlighted the importance of minerals on *L. plantarum K16* microbial cell growth (Figure 28).

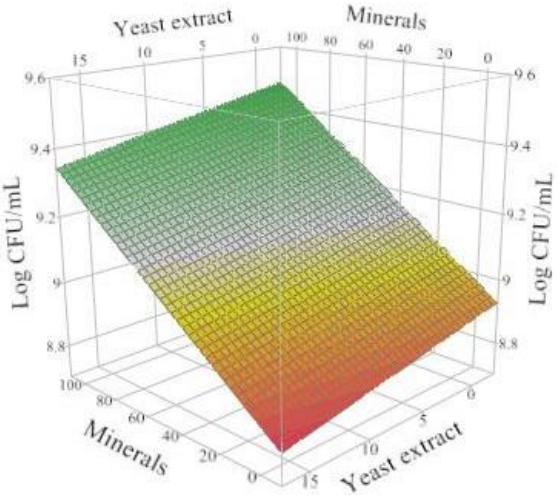


Figure 28: Response surface matrix representing the combined effect of yeast extract and minerals concentrations on the *L. plantarum K16* microbial growth using tomato by-product as fermentation medium. Glucose concentration of 12.5 g/L.

As Table 4 shows, independently of yeast extract concentration, the microbial growth was maintained at around 8.5 log CFU/mL. Still, at any concentration point, the increased concentration of minerals significantly increased ($P \leq 0.05$) the microbial growth, reaching a value of 9.5 log CFU/mL.

According to these results, the predicted model that the optimal growth of *L. plantarum K16* was expected around 9.53 log CFU/mL using 25 g/L of glucose, 12 g/L of yeast extract and 100% of minerals supplementing the tomato by-product. In these fermentation conditions, the growth of *L.plantarum K16* is expected to have a higher yield than the growth obtained using commercial MRS broth, where the greatest microbial growth was 9.11 ± 0.09 log CFU/mL after 24 h of fermentation. In this regard, Manzoor et al. (2017) reported that *L. plantarum AS-14* strain had greater growth yield in a low cost media, composed of 60 g/L of cheese whey, 15 g/L of glucose and 15 g/L of corn steep liquor, producing 10.23 log CFU/mL compared to 9.90 log CFU/mL using MRS broth.

4.5.2 Gamma-aminobutyric acid production using tomato by-product

The production GABA in tomato by-product was assessed using a Box-Behnken experimental design. According to the results obtained in the previous fermentation trials, different concentrations of glucose (20, 25 and 30 g/L), yeast extract (4, 8 and 12 g/L) and MSG (350, 450 and 550 mM) were combined to determine the best conditions to get the greatest GABA yield after 96 h of fermentation. The following equation was obtained by considering the parameters that mainly influence the production of GABA by *L. plantarum K16*:

$$Y = 1153 - 132.94A + 192.23B + 153.86C - 110.83AB + 136.33BC - 95.32A^2$$

where, Y is the predicted GABA yield, A is glucose concentration, B the yeast extract concentration, and C the MSG concentration. This equation was used to calculate the predicted GABA yield and the absolute deviation observed between the experimental data and the predictive GABA yield to ensure its reliability (Table 6).

Table 6: Experimental and predicted gamma-aminobutyric acid (GABA) yield (mg/mL) produced by *L. plantarum K16* obtained from a Box-Behnken experimental design combining different concentrations of glucose (g/L), yeast extract (g/L) and MSG (mM) using tomato by-product as fermentation medium. AD, absolute deviation between experimental and predicted GABA yield.

Glucose concentration	***Yeast extract concentration***	***MSG concentration***	***Experimental GABA yield***	***Predicted GABA yield***	***AD***
25	12	350	1107.91	1004.20	103.71
20	4	450	975.12	1042.87	67.74
30	4	450	860.33	776.98	83.35
25	4	350	929.35	892.41	36.95
30	8	350	736.08	815.36	79.28
30	8	550	1146.17	1123.08	23.10
30	12	450	956.36	1161.45	205.09
25	8	450	1248.43	1102.00	146.43
25	8	450	1092.40	1102.00	9.60
20	8	350	951.11	1081.24	130.13
25	4	550	881.75	927.45	45.70
20	12	450	1514.48	1427.33	87.15
25	12	550	1605.64	1584.58	21.06
20	8	550	1321.74	1388.95	67.21
25	8	450	1205.51	1102.16	103.36

The suitability of the predictive model was evaluated by looking at the R^2 and the adjusted R^2, which were 0.94 and 0.89, respectively. As well, the ANOVA analysis showed in Table 7, supported the significant relationship between the variables and the response observed in the actual model.

The suitability of the predictive model was evaluated by looking at the determination coefficient (R^2) and the adjusted R^2, which were 0.80 and 0.69, respectively. The significant relationship between the independient variables and the GABA yield observed in the model was evaluated by ANOVA. The statistical results showed that the concentrations of glucose, yeast extract and MSG supplied to the fermentation medium significantly affected ($P \leq 0.05$) the production of GABA (Table 7).

Table 7: Results of the ANOVA for the independient variables included in the predictive model for GABA production of L. plantarum K16 strain using tomato by-product as fermentation medium

Source	*Sum of Square*	F-value	*P*
A	141381.69	21.25	0.002
B	295618.98	44.43	0.000
C	189373.97	28.46	0.001
AB	49135.37	7.38	0.026
BC	74346.20	11.17	0.010
A^2	33924.13	5.10	0.054

A=Glucose (g/L); *B*=Yeast (g/L); *C*=MSG (mM)

As in case of microbial growth, three-dimensional response surface curves were plotted to show how GABA production was affected by the combintaion of different concentrations of two independent variables, maintainig the third independient variable fixed on its central point value. Figure 29 depicts the variation of GABA production according to the combination of the different yeast extract and glucose concentrations, with a fixed concentration of 450 mM of MSG.

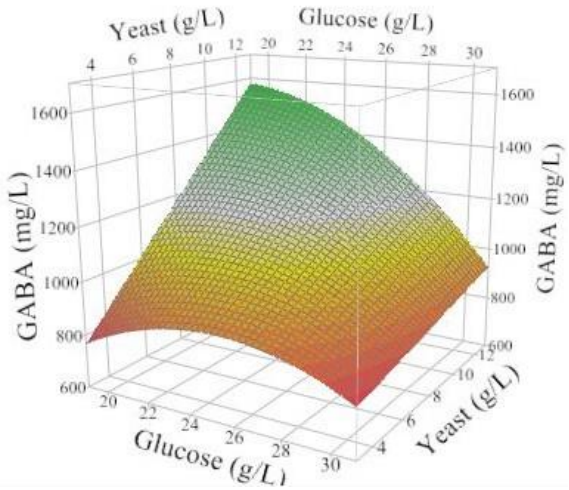


Figure 29: Response surface matrix representing the combined effect of yeast extract and glucose concentrations on the production of gamma-aminobutyric acid (GABA) by *L. plantarum K16* using tomato by-product as fermentation medium. Monosodium glutamate concentration of 450 mM.

The response surface matrix showed that GABA production increased at the same time as the concentration of yeast extract increased while the glucose concentration decreased. For instance, with 20 g/L of glucose and 4 g/L of yeast extract, and 450 mM of MSG, the GABA production was 975.12 mg/L (Table 6). At the same concentration

of glucose and MSG but with the addition of 12 g/L of yeast extract, the GABA yield significantly increased ($P \leq 0.05$) to 1514.48 mg/L. However, maintaining 450 mM of MSG and raising the glucose concentration up to 30 g/L with 4 g/L of yeast extract the GABA yield dropped to 860.33 mg/L, and this yield slightly increased to 956.36 mg/L when 12 g/L of yeast extract was added. Thus, glucose concentration was inversely correlated with GABA production. For example, 12 g/L of yeast extract, 450 mM of MSG and 30 g/L of glucose resulted in 956.36 mg/L of GABA, which significantly increased ($P \leq 0.05$) to 1514.48 mg/L of GABA reducing the concentration of glucose to 20 g/L.

Likewise, Figure 30 the response surface matrix corresponding to the combined effect of the concentrions of glucose and MSG while the yeast concentration kept constant (8 g/L) shows that GABA production increased when the concentration of MSG raised and at the same time that of glucose decreased.

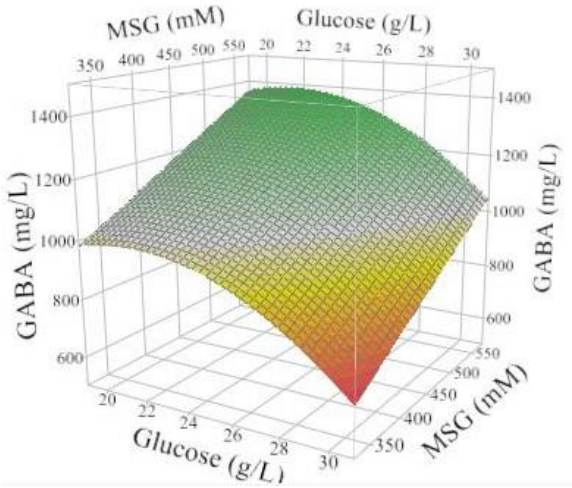


Figure 30: Response surface matrix representing the combined effect of monosodium glutamate (MSG) and glucose concentrations on the production of gamma-aminobutyric acid (GABA) by *L. plantarum K16* using tomato by-product as fermentation medium. Yeast extract concentration of 8 g/L.

This effect was observed in Table 6 showing that the greatest GABA yield (1321.74 mg/L) was achieved using 20 g/L of glucose, 8 g/L of yeast extract and 550 mM MSG. The response surface matrix that evaluated the relationship between yeast extract and MSG concentrations with a constant glucose concentration of 25 g/L showed that the highest GABA yield was produced with a simultaneous increase of MSG and yeast extract concentrations (Figure 31). For instance, Table 6 indicates that 929.35 mg/L of GABA was produced using 25 g/L of glucose, 4 g/L of yeast extract and 350 mM MSG.

Maintaining the same concentration of glucose, an increase of yeast extract concentration up to 8 g/L and 450 mM of MSG broadly increased the amount of GABA (1248.43 mg/L). Moreover, the GABA production was significantly increased ($P \leq 0.05$) up to 1605.64 mg/L when using 12 g/L and 550 mM of yeast extract and MSG, respectively.

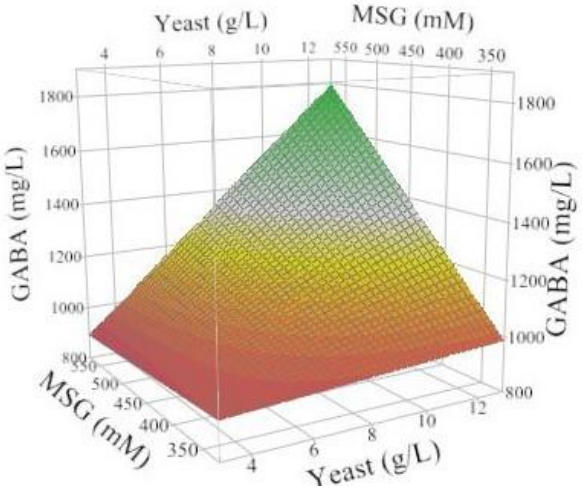


Figure 31: Response surface matrix representing the combined effect of monosodium glutamate (MSG) and yeast extract concentrations on the production of gamma-aminobutyric acid (GABA) by *L. plantarum K16* using tomato by-product as fermentation medium. Glucose concentration of 25 g/L.

According to these results, the model predicted that the optimal GABA production could be expected around 1783.86 mg/L by adding to the tomato by-product 20 g/L of glucose, 12 g/L of yeast extract and 550mM of MSG. Therefore, this predictive model was useful to confirm that a lower concentration of sugars (glucose) coupled with a higher concentration of protein source (yeast extract) and L-Glu (MSG) were needed to enhance the production of GABA by *L. plantarum K16* using tomato by-product as fermentation medium

Moreover, the data obtained in the previous fermentation trials were introduced in the model and the results showed that 1490.32 mg/L of GABA were expected to be produced using a concentration of 25 g/L of glucose, 12 g/L of yeast extract and 500 mM of MSG. This predicted amount of GABA was lower than the experimental concentration obtained by *L. plantarum K16* (1776.75 mg/L). Hence, these fermentation conditions were selected as the most suitable to develop of the new functional ingredient. Other studies have optimised the production of GABA using different by-products with the potential to develop new formulations in food or pharmaceutical industry. For example,

Falah et al. (2021) produced 300 mg/L of GABA by *L. brevis PML1* strain using 14.77% of dairy sludge, 6.27% soybean meal, and 0.49% of ammonium sulfate. Falah et al. (2022) used 29.27% of dairy sludge, 24.77% of molasses and 10.49% of soybean meal to obtained 359.45 mg/L of GABA produced by *L. fermentum.*

4.6. *In vitro* gastrointestinal evaluation of *Lactiplantibacillus plantarum K16* and gamma-aminobutyric acid

The digestion process is an intricate physicochemical bonded group of reactions focused on breaking complex matrices to enhance nutrient absorption (National Institute of Diabetes and Digestive and Kidney diseases, 2018). Gastrointestinal tract is characterised due to extreme conditions such as acid pH and high concentrations of salts that enhance the break down of food. This process starts with food ingestion, then it moves to the acidic environment (pH 1-3) of the stomach, where it could stay there from 5 min to 2 h (Figure 32).

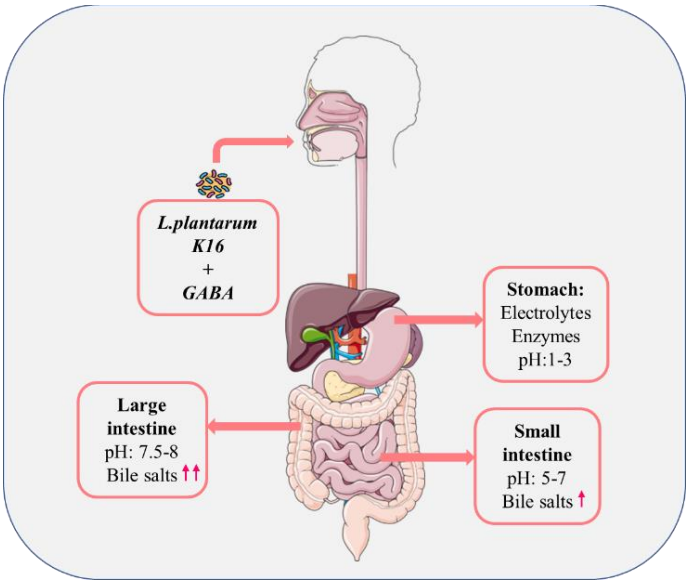


Figure 32: Schematic picture of the gastrointestinal aparatus highlighting the main biochemical and physic-chemical conditions.

Here, the viability of the probiotic and the integrity of bioactive metabolites such as GABA could be threatened by the mechanic movements of the stomach, the hydrolytic enzymes such as pepsin, and the high concentration of electrolytes like sodium, potassium or calcium and mucus (Sensoy, 2021). Boland et al. (2014) reported that during food processing in the stomach, the pH changes constantly, since at the beginning it is between 1 to 3, then it goes to 5.5 to 7, and ends with a pH of 4-5. Afterwards, the food goes

through the pylorus and arrives in the small intestine where the pH is neutralized up to 5-7, and the concentrations of pancreatic juice and bile salts increase (Figure 32). In this case, the change in pH coupled with the high concentration of bile salts and digestive enzymes could harm the probiotic cell membrane and affect the postbiotic stability (Han et al., 2021). Finally, the probiotic and GABA would move to the large intestine, an anaerobic environment inhabited by approximately 10^{12} CFU/mL microorganisms that absorb nutrients, metabolise bile salts, enzymes, and undigested compounds, and produce vitamins and SCFA. In the large intestine, mainly in the colon, commensal microorganisms could hinder the adhesion and establishment of probiotics (Ouwehand & Salminen, 2003).

Functional ingredients (probiotic and GABA) mainly have a beneficial effect on the intestine. Thus, the bioactive compounds and the probiotic microorganisms present in these functional ingredients must preserve their stability and viability through the gastrointestinal tract to perform a beneficial effect (Syngai et al., 2016). Therefore, the viability of *L. plantarum K16* and the stability of GABA against gastrointestinal conditions was assessed through an *in vitro* assay.

The resistance of *L. plantarum K16* and GABA against gastrointestinal conditions was independently studied by mimicking gastric juice (pepsin and salts) at pH 2, 4 and 6, and bile juice (bile extract and salts) at pH 7.5 during 120 min. The viability of *L. plantarum K16* in the gastric juice at pH 2 was maintained for 90 min over 99%. Nevertheless, after 120 min, the viability of *L. plantarum K16* strain significantly decline ($P \leq 0.05$) by around 4%. However, when the gastric pH was between 4 and 6, the viability of *L. plantarum K16* was not significantly reduced ($P > 0.05$) after 120 min. Similarly, the viability of *L. plantarum K16* persisted stable after 120 min in contact with the intestinal juice (Figure 33).

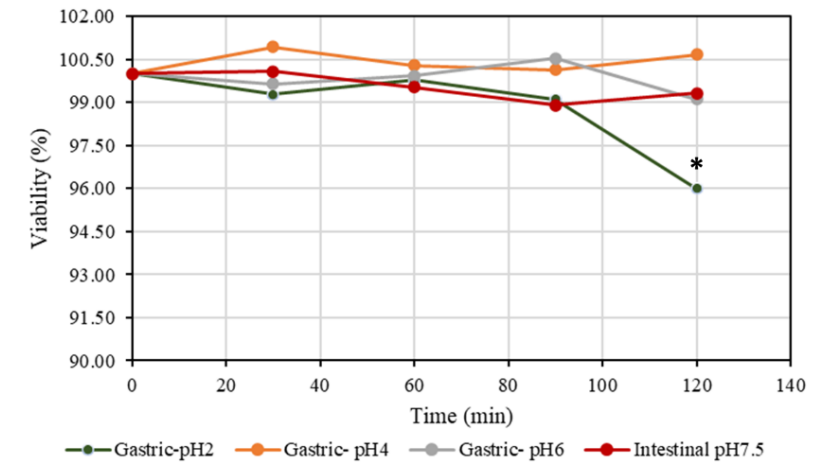


Figure 33: Viability of the growth of *L. plantarum K16* strain under gastric and intestinal conditions during 120 min. *, significant differences ($P \leq 0.05$) among fermentation times for each gastric and intestinal condition.

Generally, bacteria like *Lactobacillus spp* can maintain a stable neutral intracellular pH under an acidic extracellular environment using several mechanisms such as proton pumps, decarboxylation and deamination pathways, modification of the cell membrane or metabolic regulation. Proton pumps, mainly F_0-F_1-adenosin triphosfatase (F_0-F1-ATPase) pumps, are considered the most important system to preserve pH homeostasis based on pumping excessive protons to the cytoplasm (Guan & Liu, 2020). Specifically, when *Lactobacillus spp* introduce extracellular protons into the cell through the F_0-F_1-ATPase pump, ATPis produced and accumulated. However, a low extracellular pH triggers the decrease of the internal pH coupled with ATP consumption decrease of available energy and, thus, reduces the cell viability (Van de Guchte et al., 2002). Papadimitriou et al. (2016) reported that F_0-F_1-ATPase activity depends on the catabolism of substrates, the demand for proton transport and the concentration of ATP available. Also, the F_0-F_1-ATPase activity is strain-related, and for instance, *L. plantarum* strains usually present optimum activity ranging between pH values 5.0 to 5.5, and lower pH values induce the decrease of their viability. Kook et al. (2019) reported that after 2 h at pH 2.5 the microbial growth of *L. plantarum BioE LPL59* strain decreased from 9.69 to 4.39 log CFU/mL. Likewise, Yu et al. (2013) isolated several *L.plantarum* strains and none of them managed to maintain viability greater than 78% under gastrointestinal conditions.

The stability of GABA under gastrointestinal conditions is showed in Figure 34. As observed, the stability of GABA was significantly reduced ($P \leq 0.05$) by different gastric and intestinal conditions.

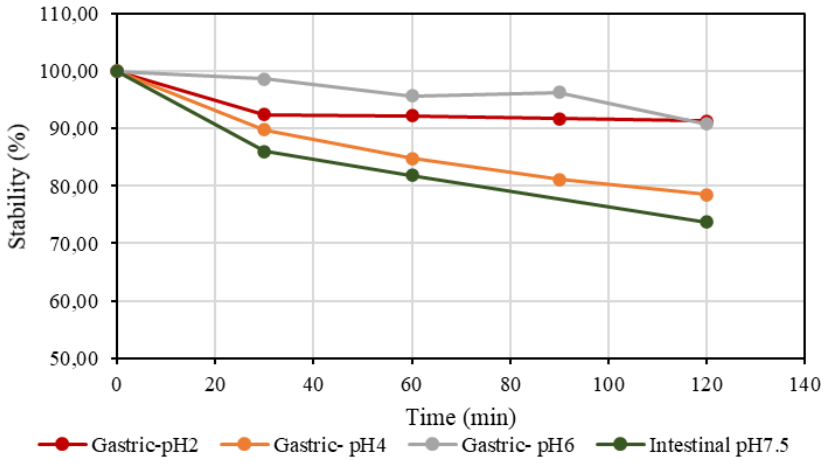


Figure 34: Stability of gamma-aminobutyric acid under gastric and intestinal conditions during 120 min. *, significant differences ($P \leq 0.05$) among fermentation times for each gastric and intestinal condition.

GABA concentration under gastric conditions at pH 2 was not significantly reduced ($P > 0.05$) after 120 min. When the gastric juice at pH 4 was tested, the GABA viability decreased significantly ($P \leq 0.05$) to 78.5% after 120 min. Likewise, the gastric juice at pH 6 reduced significantly ($P \leq 0.05$) the GABA viabilityto 91% after120 min. Turning to intestinal juice evaluation, GABA viability was significantly decreased ($P \leq 0.05$) to 74% after 120 min of digestion (Figure 34). Le et al. (2020) evaluated the stability of GABA using solutions of pH 2, 4, 6.5 and 8 without heating. In all the cases, the concentration of GABA was stable but when gastric conditions were simulated using pH 2 at 37 ºC, the viability of GABA was lost by around 31%. Furthermore, Khan et al. (2015) studied the stability of GABA in fermented rice under different pH values and they observed that GABA was more stable using pH 4 or pH 6 than in fermented rice at pH 2 or pH 7.5.

In short, the overall evaluation of the *in vitro* gastrointestinal assessment of *L. plantarum K16* and GABA indicated that the gastrointestinal conditions could negatively affect the viability of the probiotic and the stability of the postbiotic metabolite. Therefore, a protective capsule was designed to ensure both probiotic and postbiotic properly arrive to the gut.

4.7. Design of a protective microcapsule for *Lactiplantibacillus plantarum K16* and gamma-aminobutyric acid

The results of this subsection are briefly explained because they are mainly included into the patent proposal (Annex III). Furthermore, the encapsulation process was conducted following several steps. In the first step, the biomass of *L. plantarum K16* was obtained by cultivating the microorganism under the previously optimised conditions using tomato by-product as fermentation medium. Other parallel fermentation was conducted to produce GABA under optimised conditions with tomato by-product. The fermented tomato by-product enriched with GABA was further clarified before using it in the encapsulation process. Then, a clarified GABA-enriched tomato by-product was mixed with the biomass of *L. plantarum K16* and, subsequently, 2% of alginate was added to create the encapsulation mixture. Alginate is an anionic unbranched heteropolysaccharide composed mainly of D-mannuronic and L-glucuronic acid, and it is considered the most widely used biopolymer for the microencapsulation of probiotic microorganisms (Pech-canul et al., 2020).

The encapsulation mixture was introduced in the vibration-jet encapsulator which is based on a continuous laminar jet cut by a vibrational frequency. In this case, the structure of the drop will be related to the viscosity of the extrusion material, the diameter of the nozzle, the velocity of the laminar jet and the frequency applied during the encapsulation process (Chávarri et al., 2012; Heinzen et al., 2004; Whelehan & Marison, 2011). Therefore, encapsulation parameters such as pressure, vibration frequency and size of the nozzle were optimised to get the best shape and size of the capsule. In this case, optimised mono-dispersed droplets were obtained using a extrusion nozzle of 200 µm, vibration frequency of 1,500 Hz and extrusion pressure of 450 mbares (Figure 35).

After the formation of droplets, alginate capsules (Figure 37a) were produced due to the ionic exchange of sodium molecules, from the L-glucuronic acid of the alginate, with the divalent calcium from the hardening bath. Calcium chloride is an idoneous gelling agent since it favours the rapid formation of spheres by the three-dimensional grouping of four L-glucuronic acid residues that generate the conformation called "egg-box structure" resulting as calcium alginate beads (Cook et al., 2012; Martín et al., 2015). In addition, the pKa values of D-mannuronic (3.38) and L-glucuronic (3.65) acid improve the preservation of the capsule structure at low

pH values. Therefore, the created alginate capsules could pass easily through the acidic gastric tract but the alkalinity of the intestine causes the capsule to break down (Chuang et al., 2017).

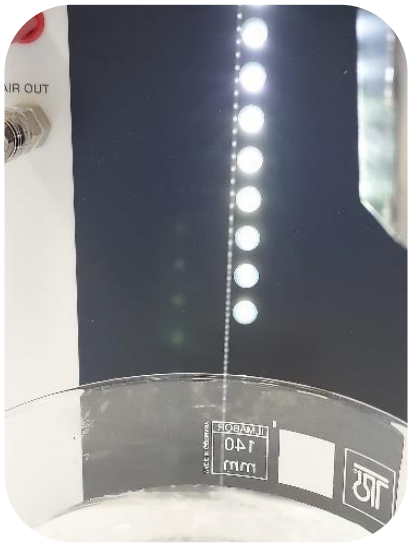


Figure 35: Picture of the droplets formation composed by 2% of alginate plus clarified gamma-amino butyric acid -enriched tomato by-product and *L. plantarum K16* strain.

The size and shape of the microcapsules obtained from the clarified fermented tomato by-product enriched with GABA and *L. plantarum K16* can be observed in Figure 36. The shape of the microcapsules was spherical and the average diameter size measured for 50 wet microcapsules was 856.08 ± 121.61 µm.

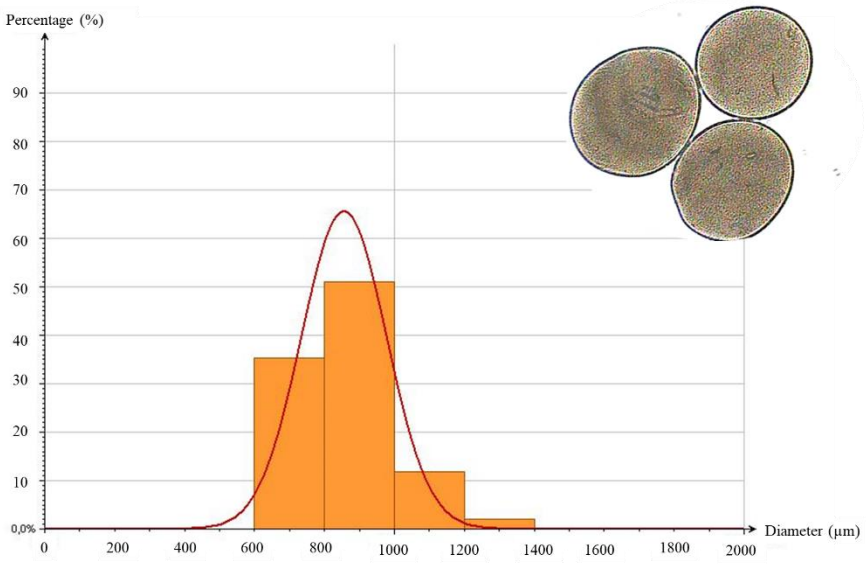


Figure 36: Shape and average diameter size (n = 50) of the 2% alginate microcapsules composed of clarified GABA-enriched tomato by-product and *L. plantarum K16* strain.

After encapsulation process, the microcapsules obtained were immersed in milk to preserve the viability of the microorganisms in the drying step (Figure 37b). Skim milk is a widely used cryoprotectant with high efficacy because milk sugars, mainly lactose, act as dehydrating agents, decreasing intracellular water and preventing cell death when temperature drops. In addition, the colloidal structure of milk acts as a protective barrier preventing microorganisms from being damaged (Jagannath et al., 2010). Chávarri et al. (2012) indicated that the skim milk prevents the cellular injury by stabilizing cell membrane constituents. Finally, microcapsules were recovered from the milk bath and were lyophilised, resulting in the final new functional ingredient composed of clarified GABA-enriched tomato by-product and *L. plantarum K16* strain (Figure 37c).

Figure 37: Photographs of (a) microcapsules after encapsulation; (b) microcapsules after recovering from milk immersion; and (c) lyophilised functional ingredient composed of clarified GABA-enriched tomato by-product and *L. plantarum K16* strain.

The lyophilised functional ingredient resistant to gastrointestinal conditions (Figure 37c) contained 9.78 ± 0.05 log CFU/g of *L. plantarum K16* and 20.18 ± 1.05 mg/g of GABA. The quantity of *L. plantarum K16* strain will be enough to confer a potential beneficial effect as the minimum probiotic concentration that should arrive at the gut is around 6 log CFU/g (Terpou et al., 2019). Sahab et al. (2020) reported that GABA could have different health effects depending on the concentration supplied. For example, a single dose from 3.6 to17.9 mg of GABA had an antihypertensive effect in rats, or the six-week intake of 6 mg/mL of a GABA drink had an anti-diabetic effect in humans. Xie et al. (2017) observed that 40 mg/Kg/d of GABA for 14 days enhanced the production of SCFA improving the colon health of Kumming mice. Furthermore, Choat et al. (2019) reported in a clinical trial that the supplementation of 200 mg of encapsulated GABA could prevent the development of type I and II diabetes in children.

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SECTION II

6. CONCLUSIONS

The research conducted in this Ph.D. Thesis has led to the following conclusions:

1. From a kimchi-fermented product, using natural raw materials, a great source of lactic acid bacteria was obtained, identifying *Lactiplantibacillus plantarum K16* strain as a probiotic capable to produce the postbiotic metabolite gamma-aminobutyric acid (GABA).
2. *In vitro* safety and probiotic characterisation studies showed that *L. plantarum K16* strain could be considered harmless and potentially promote human health by metabolising different nutrients and inhibiting common pathogens such as *Salmonella typhimurium*.
3. Fermentation parameters, such as incubation temperature, inoculum, initial pH, nutrients concentration, and fermentation time, significantly impacted *L. plantarum K16* for GABA production using commercial Man Rogosa Sharpe (MRS) broth. *L. plantarum K16* achieved the production of more than 2100 mg/L of GABA applying optimal fermentation conditions using commercial MRS broth.
4. Among the agri-food by-products revaluated, tomato by-product was selected as the best fermentation media to develop the functional ingredient due to the high GABA yield (higher than 1775 mg/L) and the great microbial cell growth (9.5 log CFU/mL) of *L. plantarum K16* strain .
5. The adequate combination of *L plantarum* and GABA allowed the development of a microencapsulated functional ingredient, resistant to gastrointestinal conditions, allowing to preserve the integrity of its components to have a beneficial effect on the intestine with impact to the systemic level.

SECTION III

ANNEX I:
PUBLICATIONS

ANNEX I.I:
PUBLICATION

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Gamma-aminobutyric acid and probiotics: Multiple health benefits and their future in the global functional food and nutraceuticals market

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ABSTRACT

Probiotics have attracted growing interest in recent decades due to their multiple health benefits. The synergistic relationship between probiotics and prebiotics can enhance the production of metabolites called postbiotics, which are gaining increasing importance because of their beneficial functions in the gastrointestinal tract and their influence on different organs and tissues. Notable among the postbiotics is gamma-aminobutyric acid, which plays an essential role in the prevention of neural disease, type 1 diabetes, cancer, immunological disorders and asthma. Generally, gamma-aminobutyric acid is produced by lactic acid bacteria, which under certain conditions can produce a high amount of this amino acid. The food industry has leveraged this capacity to develop functional foods enriched with gamma-aminobutyric acid.

1. Probiotics and their beneficial health effects

1.1. Development of the concept of probiotics

Probiotics are generally defined as "live microorganisms that when administered in adequate amounts are able to provide benefits to the health of the consumer" (FAO/WHO, 2006). Microorganisms used as probiotics are classified as GRAS (generally regarded as safe), which are characterised by a very low probability of infection. These microorganisms must be capable of withstanding the acidic conditions of the stomach and the high concentration of bile acids present in the small intestine (Nagpal et al., 2012).

The concept of probiotics is not new, but has changed over the years, even in the new millennium. The probiotic products developed by the pharmaceutical industry have become increasingly popular among the public due to their beneficial effects demonstrated in human research, prompting an increase in the consumption of yoghurt and fermented milks and generating wider acceptance in the medical community, health institutions and consumers.

In 2014, PubMed indexed 1,800 articles under the term probiotics, double the number of those indexed in 2007 (820 articles), which in turn was ten times higher than in 1999, when only 172 articles were reported. These figures reflect the expansion and importance of probiotics (Linares et al., 2016).

The definition of the term probiotic has been much discussed and has changed over the course of the last 50 years. The most important definitions are reviewed below:

- In 1974, Parker postulated the term as it is known today, defining it as "organisms and substances that contribute to intestinal microbial balance" (Parker, 1974). This concept was modified by Fuller in 1991 (Fuller, 1991) and by Salminen in 1996 Salminen, 1996). The decade of the 1990s was considered "the age of probiotics" (Castañeda-Guillot, 2014) and the concept continued to expand in subsequent years.
- In 2001, a group of international scientists met at the request of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) to discuss the emerging issue of probiotics (FAO/WHO, 2006). Revision of the term resulted in the following definition: "live microorganisms that when administered

Abbreviations: CAGR, compound annual growth rate; CNS, central nervous system; EFSA, European Food Safety Authority; FAO, Food and Agriculture Organization; FDA, Food and Drug Administration; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; GI, gastrointestinal; Glu, L-glutamic acid; GRAS, generally regarded as safe; IgA, immunoglobulin A; LAB, lactic acid bacteria; MSG, monosodium glutamate; OCD, obsessive-compulsive disorder; PLP, pyridoxal 5-phosphate; PTSD, post-traumatic stress disorder; Puu, putrescine; SCFA, short-chain fatty acid; TCA, tricarboxylic acid cycle; WHO, World Health Organization

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in adequate amounts confer a benefit to the health of the host". This became the approved and most widely accepted concept worldwide. The following year, in 2002, a FAO/WHO Working Group developed guidelines to assist in the interpretation of the original document (Quinto et al., 2014).

- In Finland, Isolauri, Kirjavainen and Salminen (2002) described probiotics as "living or inactivated microbes that have documented effects in reducing the risk of disease or as a coadjuvant treatment" (Kleinman et al., 2018).
- In 2013, the International Scientific Association of Probiotics and Prebiotics convened a group of international experts in various scientific and medical fields, including gastroenterologists, paediatricians, family physicians, clinicians, microbiologists, pharmacologists, geneticists, immunologists, nutritionists and researchers in the pharmaceutical industry related to probiotics, to carry out a new analysis of probiotics with the aim of establishing consensus on their use and the terminology to employ, which they conceptualised as follows: "oral probiotics are live microorganisms that after their ingestion in a specific number, exert benefits for the health of the host, beyond those that are inherent in basic nutrition". This definition, although quite similar to that of the WHO/FAO, was more accurate in guiding the medical community and consumers. This is the most recent definition to be established (Valdovinos et al., 2017).
- In 2017, the World Organisation of Gastroenterology reviewed the definition and maintained that of the FAO/WHO in 2001, stating: "Probiotics are living microorganisms that, when administered in adequate amounts, confer a benefit to the health of the host" (Guarner et al., 2017).

1.2. The health potential of probiotics

As can be seen, various definitions have been formulated for the term probiotic, but they all bear some relation to the following characteristics (Hill et al., 2014): (1) a probiotic agent must show non-pathogenic properties; (2) ability to survive in the digestive tract; (3) adherence to the intestinal epithelium; (4) colonisation of the intestinal tract; (5) production of antimicrobial substances; and (6) adequate survival (stability) in the form of powder, liquid or food.

The microorganisms most commonly used as probiotics belong to the genera *Lactobacillus*, which is classified as lactic acid bacteria (LAB), and *Bifidobacterium* (Georgieva et al., 2014). Other LAB such as *Lactococcus, Enterococcus, Streptococcus* and *Leuconostoc* are also classified as probiotics. In addition, some fungi and yeasts of the genus *Aspergillus* and *Saccharomyces* can be considered probiotics (Amara & Shibl, 2015; Kechagia et al., 2013).

Consumption of probiotics favours the maintenance of a healthy intestinal microbiota via several different mechanisms of action, such as preventing pathogen adhesion or colonisation (Zhang et al., 2019), as well as during antibiotic treatment (Valdés-Varela et al., 2016). Other important mechanisms of action of probiotics include the production of metabolites called postbiotics, which are potentially beneficial to health (Kerry et al., 2018), and modulation of the immune system by probiotics called immunobiotics (Villena & Kitazawa, 2017). These beneficial effects will be explained in depth below.

Among the beneficial effects of probiotics considered therapeutic are the following:

- Antagonistic action against pathogenic microorganisms (Linares et al., 2016; Sotoudegan et al., 2019; Tsiouris & Tsiouri, 2017). The most important action of the gut microbiota is unquestionably to protect against infection and colonisation of the digestive tract by pathogenic microorganisms. The mechanisms that form the host's first line of defence against intestinal infection are called resistance to colonisation, competitive exclusion and the barrier effect. Pathogenic microorganisms can be suppressed in several ways:
  - Organic acids (e.g. lactic or acetic acid) produced from food carbohydrates lower the pH and limit the development of *Escherichia coli* and species of the genus *Salmonella* (Rahimzadeh, Dolatabad, & Rostami, 2014; Rahimzadeh, Fazeli, Mozafari, & Mesbahi, 2015). In addition, acidification of the digestive tract seems to promote intestinal peristalsis.
  - Probiotics appear to suppress the growth of pathogenic bacteria by producing bacteriocins, antimicrobial substances that inhibit the pathogens that often cause infections (Tsiouris & Tsiouri, 2017).
  - Probiotic strains present a high capacity for interaction with mucosal and epithelial surfaces, enabling their adhesion and preventing pathogen colonisation (Zhang et al., 2019). Valdés-Varela et al. (2016) conducted a study analysing the effect of different types of *Bifidobacterium* on colonisation by *Clostridium difficile*. Following antibiotic treatment, *Clostridium difficile* normally occupies free niches in the intestine, triggering diarrhoea of varying degrees of severity. However, these authors found that after administration of the probiotics *Bifidobacterium longum* or *Bifidobacterium breve*, the amount of *Clostridium difficile* decreased because the probiotic bacteria occupied the free niches and displaced the pathogenic bacteria. In addition, the probiotics exerted a competitive inhibition effect by consuming nutrients, thus rendering these unavailable to pathogenic bacteria and helping prevent colonisation by undesirable microorganisms.
- Stimulation of immunity (Aureli et al., 2011; Cerbo, Palmieri, Aponte, Morales-Medina, & Iannitti, 2016). One of the notable characteristics of the intestinal microbiota is its capacity to stimulate and regulate the innate and adaptive immune response. The microbiota intervenes in the innate immune response, which consists of protective barriers, phagocytes and natural killer cells, as well as the adaptive or acquired immune response, composed of T and B lymphocytes. Depending on the pathology, the immune system will activate one or the other response (Mishra & Mishra, 2018).

  Probiotic strains have a stimulating action on the host's immune system, acting both on the cells involved in natural immunity and on those related to specific immunity, and also activating macrophages. Although the full mechanisms have not yet been elucidated, it is known that only microorganisms capable of surviving in the gastrointestinal (GI) tract can activate macrophages (Dong, Rowland, & Yaqoob, 2012; Miller, Lehtoranta, & Lehtinen, 2019). In addition, it seems that the presence of probiotic microorganisms favours antibody production, especially secretory immunoglobulin A (IgA) in the intestinal lumen, which can inhibit the adherence of pathogenic bacteria to the mucosal surface:
  - Causing the agglutination of bacteria.
  - Modifying the adhesion factors present on the surface of the bacteria.
  - Interfering with adhesin-receptor interactions.

  Due to their action on the immune system, LAB have the potential to prevent intestinal infections, protect against damage related to the immune system and act as immunomodulators (Miller et al., 2019).
- Neutralisation of toxic products (Sotoudegan et al., 2019). Inactivation of toxic compounds is another very important aspect of probiotic action. It seems that probiotics attenuate intradigestive catabolism, orienting liver function. They accumulate in the gut microbiota where they reduce the absorption of toxic substances such as ammonia, amines and indole. It also seems that they reduce the biotransformation of bile salts and fatty acids into toxic products.
- Modulation of stress (Novik & Savich, 2019). Stress is one of the factors that influence variations in the gut microbiota. Stress alters digestive physiology, increasing peristalsis and secretions of HCl and mucus in the digestive tract, and thus modifying the microbiota and the activities that depend on it.

- Protection of the urogenital system (Cerbo et al., 2016). In healthy women, the urogenital system is characterised by a complex microbiota whose equilibrium undergoes numerous fluctuations. Multiple studies have confirmed that endogenous *Lactobacillus* play a similar role in the prevention of infection in the urogenital system as they do in the intestine.
- Bacterial overgrowth, intestinal motility disorders and intestinal microbiota (Sotoudegan et al., 2019). Bacterial overgrowth syndrome is defined as abnormal bacterial proliferation in the small intestine, generally due to the previous existence of anatomical alterations or poor intestinal motility. In most cases, it only causes mild nonspecific symptoms such as prolonged diarrhoea, flatulence and abdominal pain. However, bacteria can damage the intestinal mucosa, leading to malabsorption syndrome which in turn leads to secondary malnutrition due to loss of nutrients. Overgrowth of Gram negative bacteria in the intestinal lumen displaces the normal microbiota of the small intestine, giving rise to a series of effects that are responsible for malabsorption symptoms. Studies of probiotic administration as adjuvant treatment constitute a promising therapeutic approach in this field.
- Implication and effects of probiotics in different diseases (Sotoudegan et al., 2019). Increasing numbers of studies have analysed intestinal microbiota variability in different inflammatory diseases of the intestine such as coeliac disease (de Sousa Moraes, Grzeskowiak, de Sales Teixeira, & do Carmo Gouveia Peluzio, 2014) and Crohn's disease (Gensollen & Blumberg, 2017). Effective modification of the gut microbiota is therefore considered a promising therapeutic approach that influences the immune response. Probiotics play an important role in modulating intestinal lymphoid tissue and exert an immunomodulatory effect; consequently, they may have a therapeutic application in some autoimmune diseases or as prophylactics.

It should be noted that GABA is mainly produced by *Bacteroides* in the gut and that *Bacteroides* is the largest group of GABA producers in the gut (Pokusaeva et al., 2017). However, this study focused on LAB because this is the group used in the food industry.

1.3. Postbiotics as beneficial metabolites produced by probiotics

Postbiotics, also known as metabiotics (Shaikh & Sreeja, 2017; Singh, Vishwakarma, & Singhal, 2018), pharmacobiotics (Aguilar-Toalá et al., 2018) or heat-killed probiotics (Hasan et al., 2019), are bioactive compounds produced by the metabolism of probiotics, mainly LAB. Several compounds found in probiotics that are released into the environment before death are also considered postbiotics. Enzymes, polysaccharides, organic acids, short-chain fatty acids (SCFA), cell surface proteins, vitamins and lipids are all examples of these metabolic products (Aguilar-Toalá et al., 2018). Table 1 lists various postbiotics and their functions. All of these metabolites called "postbiotics" exert a functional effect on the microbiota and are capable of modulating human health (Bolca, Van de Wiele, & Possemiers, 2013; Klemashevich et al., 2014). Fig. 1 details how probiotics metabolise different compounds to yield each postbiotic, the influence of postbiotics on the GI tract and the impact of these compounds on different organs and tissues.

Some of the postbiotics produced in the intestinal microbiota include metabolites such as GABA from L-glutamic acid (Glu), SCFAs from carbohydrates, indole from amino acids and polyphenolic acids and other functional compounds obtained from the diet (Chaluvadi, Hotchkiss, & Yam, 2016; Klemashevich et al., 2014). Thus, it has been shown that compounds derived from amino acids transformed by the intestinal microbiota are a potential class of postbiotics. For example, a possible link has been described between indole, a compound derived from tryptophan, and microbiota dysbiosis, based on evidence obtained in studies of patients with ulcerative colitis, who presented low concentrations of indole in faecal samples (Nemoto et al., 2012). In addition, Bansal, Alaniz, Wood, and Jayaraman (2010) have reported that indole decreases inflammation indicators, proinflammatory transcription factors and pathogen colonisation in intestinal epithelial cells, while increasing the strength of tight junctions and mucin production, thus demonstrating that indole is a postbiotic molecule. The SCFAs produced by the microbiota are another type of bioactive compound with a beneficial effect. In a study comparing the colon microbiota and its metabolites in people of African origin with a high and low risk of colon cancer, significant correlations were found between reduced SCFA production, higher levels of bile acid metabolites of bacterial origin and an increased risk of colon cancer (Ou et al., 2013).

Several studies have evidenced the importance of SCFAs produced by bacteria and have described the influence of these compounds on GI physiology. For example, deteriorating health in elderly patients has been related to changes in the amount of SCFAs (Claesson et al., 2012).

Currently, the mechanisms involved in the beneficial health effects of postbiotics are not entirely clear. The data available indicate that these compounds have different functional properties, for instance exerting antioxidant, antimicrobial and immunomodulatory effects. One example is the capacity of *Lactococcus lactis* MTCC 440 to synthesise nisin, a bacteriocin with antimicrobial activity (Khalighi, Behdani, & Kouhestani, 2016). Nisin inhibits the growth of potentially pathogenic bacteria such as *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus circus* (Malathi & Selvakumar, 2016).

Another example is the capacity to produce SCFAs, the most common being acetate, butyrate and propionate (Singh et al., 2018). Butyrate is used by colon epithelial cells, propionate stimulates ATP production in the liver and acetate is used by muscle cells. Nagpal et al. (2018) have analysed the mechanisms whereby different probiotic bacteria from the genera *Lactobacillus* and *Enterococcus* increase the production of SCFAs such as propionate and butyrate. Their results indicate that use of these probiotics could be beneficial for patients with diabetes, cancer, obesity or autoimmune disorders since SCFA production is reduced in these diseases (Mesnage, Antoniou, Tsoukalas, Goulielmos, & Tsatsakis, 2018).

2. Gamma-aminobutyric acid

2.1. The importance of GABA

GABA is an amino acid with a non-protein structure which is mainly produced by plants, animals and microorganisms (Lim, Cha, Lee, & Seo, 2016), and it performs different functions depending on the producer organism (Xu, Wei, & Liu, 2017). For instance, GABA is a well-known inhibitory neurotransmitter in the central nervous system (CNS) of animals (Walls, Waagepetersen, Bak, Schousboe, & Sonnewald, 2015), but in plants and microorganisms it is synthesised as a protective mechanism against stress (Xu et al., 2017).

GABA has aroused increasing research interest in the field of biotechnology due, for example, to its importance in the synthesis of nylon 4, which is considered a potential biodegradable polymer (Pham, Somasundaram, Lee, Park, & Hong, 2016), and its involvement in bioremediation of acid mine drainage. As the biosynthetic pathway of this molecule is considered an essential mechanism to protect against low pH stress, it represents a promising alternative to the addition of chemicals to neutralise acidic environments (Liu, Tang, Lin, & Xu, 2015).

However, it is the pharmaceutical and food industries which have predominated in biotechnology research, conducting extensive studies to develop GABA-rich food supplements (Boonstra et al., 2015) and fermented foods (Selhub, Logan, & Bested, 2014) which leverage the manifold health benefits of this amino acid (Sharon et al., 2014), including gut modulation (Auteri, Zizzo, & Serio, 2015), neurostimulation (Lim et al., 2018) and cardioprotection (Kim, Park, Kang, & Ji, 2014).

Table 1
List of the different functions and examples of postbiotics.

Postbiotics	Features	References
Short chain fatty acids	Function: regulation of cellular physiology and energy source. Examples: acetate, butyrate, propionate.	Shaikh and Sreeja (2017)
Vitamins	Function: metabolism stimulation. Examples: folates, biotin, riboflavin.	Singh et al. (2018)
Mediators of inflammation	Function: degradation of proinflammatory cytokines, helping attenuate inflammatory response. Example: lactocepin.	Eppinga et al. (2014)
Bacteriocins	Function: elimination of pathogenic microorganisms such as *Salmonella*, *Shigella*, *Proteous*, *Clostridium* and *Pseudomonas*. Examples: nisin, glycocin, streptolysin.	Cicenia et al. (2014), Alvarez-Sieiro et al. (2016)
Polycationic molecules	Function: regulation of adhesion and cellular immune response. Examples: polyamines such as putrescine, spermidine.	Singh et al. (2018)
Regulatory molecules of homeostasis	Function: mucin secretion to preserve structure and compounds to prevent apoptosis and promote enterocyte development. Examples: proteins p40, p75.	Bäuerl, Pérez-Martínez, Yan, Polk and Monedero (2011), Cicenia et al. (2014)
Neurotransmitters	Function: production of compounds intervening in neuronal system regulation. Examples: gamma-aminobutyric acid (GABA), serotonin, acetylcholine, histamine.	Singh et al. (2018)

Initially, chemical synthesis was used to meet the demand for GABA, but subsequent use of microorganisms to produce this compound has replaced the chemical process due to the higher yields, lower costs and lower environmental impact of the biosynthetic process (Zhao et al., 2014).

2.2. Biosynthesis of GABA in microorganisms

In terms of microbial species, either the putrescine (Puu) or glutamate decarboxylase (GAD) pathways (Jorge, Leggewie, & Wendisch, 2016) can be used to biosynthesise GABA. Fig. 2 shows each step of both pathways.

2.2.1. Putrescine pathway

The Puu pathway is a minor route used by some microorganisms to obtain GABA. The process begins with the transportation of Puu into the cell by an antiporter codified by a *PuuP* gene. Then, the Puu route takes two different paths (Kurihara et al., 2008; Rocha & Wilson, 2018). One of them begins with the transformation of Puu to γ-glutamyl-Puu. This bioconversion is carried out by a γ-glutamate-putrescine-synthetase, which is encoded by a *PuuA* gene. Subsequently, two successive oxidations are performed by a γ-Glutamyl-oxidase and a γ-glutamyl-γ-butyraldehyde dehydrogenase, codified by a *PuuB* and a *PuuC* gene, respectively (Wu, Tun, Law, Khafipour, & Shah, 2017). As a result, a γ-Glu-GABA is obtained, and then a γ-Glu-GABA hydrolase (*PuuD* gene) enhances disruption of the molecule into GABA. Afterwards, GABA can be degraded to succinate for metabolism in the Krebs cycle (Kumar,

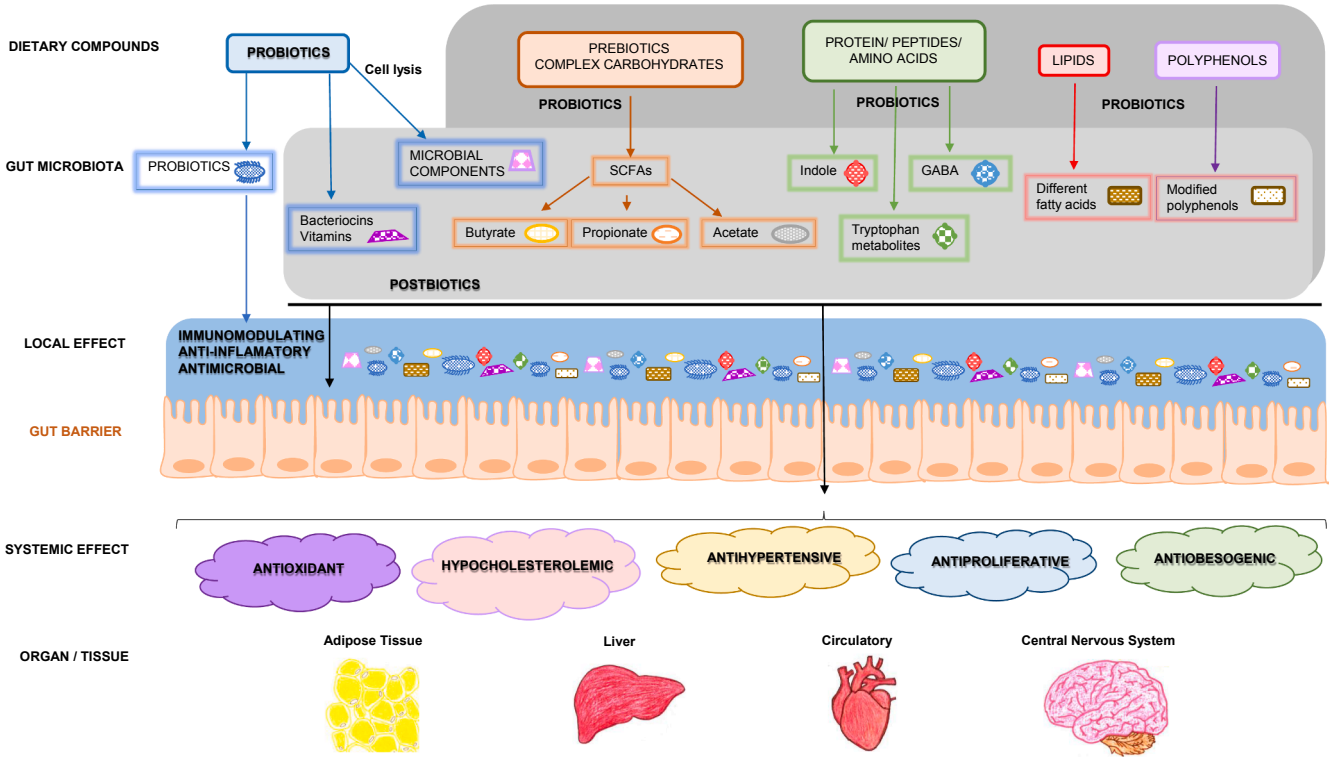

Fig. 1. Overview of the interplay between dietary components, gut microbiota, postbiotics, prebiotics and host health. Dietary compounds cause changes in the composition and activity of the intestinal microbiota, generating secondary metabolites that modulate host responses. These metabolites have a local effect on the gut mucosa and when crossing the intestinal barrier, they have systemic effects that help prevent the development of diseases.

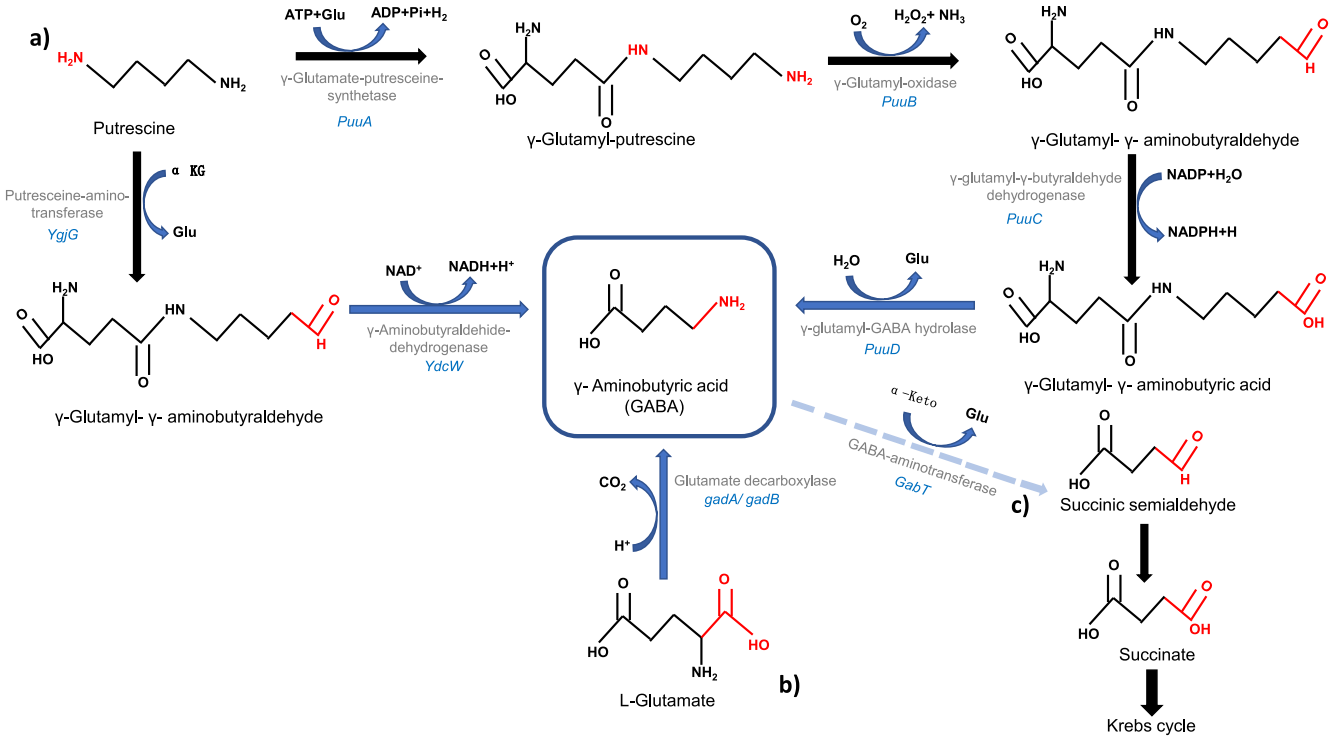

Fig. 2. Scheme of the microbial biosynthetic pathway of Gamma-aminobutyric acid (GABA) (the genes involved in each step are represented in light blue and the enzymes that are encoded by these genes are coloured in grey): (a) Putrescine pathway (b) Glutamic acid decarboxylation pathway (c) Degradation route of GABA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Saragadam, & Punekar, 2015). In the other, Puu is degraded by direct conversion to γ-aminobutyraldehyde catalysed by a Puu-amino-transferase (*YgjG* gene) and subsequent oxidation to GABA by a γ-amino-butyraldehyde-dehydrogenase (*YdcW* gene) (Kusano & Suzuki, 2015).

This pathway is not commonly found in *Lactobacillus* or *Bifidobacterium* strains (Wu et al., 2017). In contrast, another well-known bacteria, *Escherichia coli* (Cha, Jeong, Rojviriya, & Kim, 2014), and a fungi, *Aspergillus oryzae*, do present this route (Akasaka et al., 2018).

2.2.2. Glutamic acid decarboxylase pathway

A wide variety of microorganisms can synthesise GABA using the GAD pathway, including *Lactobacillus spp*. (Das & Goyal, 2015), *Escherichia coli* (Yu, Ren, Wang, & Huang, 2019), *Listeria monocytogenes* (Huang, Mao, Ji, & Alati, 2014) and *Aspergillus oryzae* (Sano, Dohmoto, & Ohashi, 2016).

The first stage of the GAD pathway is carried out by a Glu/GABA antiporter, codified by a *gadC* gene (Gao et al., 2019). This antiporter pumps the precursor Glu or its monosodium glutamate (MSG) into the microorganism (Choi et al., 2015). Then, a GAD enzyme dependent on pyridoxal-5-phosphate (PLP) catalyses transformation of the precursor to GABA which is subsequently exported to the extracellular matrix by the action of the Glu/GABA antiporter (Shi et al., 2014; Villegas et al., 2016).

The GAD enzyme is generally codified by a *gadB* gene which consists of six repetitive subunits composed of a conserved lysine residue that binds to PLP (Yu et al., 2019). According to Yunes et al. (2016), in most of the *Lactobacillus* strains (*L. rhamnosus, L. plantarum, L. casei* and *L. sakei*) GAD is encoded by a *gadB* gene. Nevertheless, *L. brevis* also possesses a *gadA* that presents a similar structure to the *gadB* gene, the only variation being in the N-terminal region. Although both genes play the same role in GAD expression, deletion of *gadB* is associated with a more marked reduction in GABA production than deletion of *gadA* (Lyu et al., 2018).

As in the Puu pathway, GABA can be degraded and introduced into the Krebs cycle. Firstly, a GABA-aminotransferase, encoded by a *gabT* gene, catalyses the biotransformation of an α-ketoglutarate into Glu (Yu et al., 2019). This reaction yields succinic semialdehyde which is subsequently converted into succinate by a succinic semialdehyde dehydrogenase codified by a *gabB* gene. Finally, the succinate enters the tricarboxylic acid cycle (TCA) (Kurihara, Kato, Asada, Kumagai, & Suzuki, 2010; Pham et al., 2016).

2.3. Production of GABA by Lactobacillus spp.

Both *Bifidobacterium* and *Lactobacillus* have received considerable attention due to their large number of GABA-producing strains (Table 2). Depending on the natural environment of each *Lactobacillus* strain, different parameters influence the expression of the *gad* genes, and thus GABA production (Lim et al., 2018).

2.3.1. Effect of environmental factors

Temperature and pH have been reported as the main environmental factors that can modulate *gad* gene expression (Lin, Li, & Qin, 2017). Shin et al. (2014) and Sa, Park, Jeong, Lee, and Kim (2015) have summarised the optimal temperatures and pH values for several *Lactobacillus* species. For example, *L. sakei* showed the highest GAD activity at 55 °C and a pH of 5, whereas 40 °C and a pH of 4.5 were the best parameters for *L. plantarum* GAD activity. Meanwhile, different strains of *L. brevis* present optimal values ranging between 30–48 °C and a pH of 4.2–5.2.

Variation in pH enhances activation of the GAD pathway since it is considered one of the special mechanisms that preserve cell homeostasis (Sanchart, Rattanaporn, Haltrich, Phukpattaranont, & Maneerat, 2017; Wang et al., 2018). Wu et al. (2017) evaluated performance of the GAD pathway in comparison with other acid resistance mechanisms, applying genetic and biochemical techniques to assay the response of *L. brevis* under acid stress. Their results confirmed that the GAD system is an essential mechanism to maintain metabolic activity under intra- and extracellular acidity.

Table 2
Lactobacillus and *Bifidobacterium* strains that produce GABA.

GABA Producer	Reference
L. plantarum	Park, Lee, and Lim (2014)
L. brevis	Binh et al. (2014)
L. sakei	Sa et al. (2015)
L. paracasei	Laureano-Melo et al. (2019)
L. bulgaricus	Gangaraju, Murty, and Prapulla (2014)
L. zymae	Park, Jeong, and Kim (2014)
L. futsaii	Sanchart et al. (2017)
L. buchneri	Zhao, Hu, Pan, and Wang (2015)
L. parbuchneri	Fröhlich-Wyder et al. (2015)
L. namurensis	Ratanaburee, Kantachote, Charernjiratrakul, and Sukhoom (2013)
L.rhamnosus	Yi Song and Yu Chui (2017)
L. fermentum	Lin et al. (2017)
B. adolescentis	Strandwitz et al. (2019)
B. adolescentis, B. longum, B. bifidum, B. breve	Yi Song and Yu Chui (2017)
B. adolescentis, B. longum, B. bifidum, B. breve; B. animalis, B. pseudolongum, B. dentium, B. thermacidophilum, B. thermophilum	Wu et al. (2017)
B. adolescentis, B. angulatum, B. dentium	Yunes et al. (2016)
B. bifidum	Kim et al. (2014)

Low intracellular pH triggers the predominance of non-charged Glu due to protonation of the γ-carboxyl group of this amino acid. Then, Glu decarboxylation consumes one proton that increases pH in the cytoplasm (Teixeira et al., 2014). Likewise, low extracellular pH subsequently decreases intracellular pH due to activation of the Glu/GABA antiporter. Acidification of the intracellular media triggers proton consumption due to bioconversion of a protonated Glu into GABA, which is transported to the extracellular media to relieve acid stress (Liu et al., 2015). Zhang, Zeng, Tan, Tang, and Xiang (2017) have analysed how initial pH affects GABA production by *L. plantarum.* The best concentration of GABA was detected at pH 5.5, obtaining double the amount of GABA yielded at pH 4.0.

Culture temperature also influences GABA production due to its relationship with GAD activation. Yang et al. (2015) have reported that GAD functionality is directly related to an increase in temperature until reaching the turning point, after which GAD activity falls to thermal inactivation. Another study with *L. plantarum* showed an increase in GAD activity up until 40 °C, obtained the highest amount of GABA at 35 °C (Shan et al., 2015). Likewise, *L. brevis* significantly increases GABA yield at 30 °C (Villegas, Brown, De Giori, & Hebert, 2016).

2.3.2. Effect of additives

GABA yield can be modulated by supplementation with several additives. The concentration of the precursor Glu or MSG strongly modifies GABA synthesis (Hasegawa, Yamane, Funato, Yoshida, & Sambongi, 2018). In addition, Tajabadi et al. (2015) have measured the relationship between the amount of GABA produced and the effect of Glu concentration in *L. plantarum* between a range of 0–600 mM, finding that GABA production increased sharply until reaching a concentration of 400 mM Glu. Meanwhile, Zhang et al. (2017) have evaluated how different MSG concentrations influence GABA production by *L. plantarum*, finding that 20 g/l was the optimal Glu concentration to obtain the best GABA results. A range between 0 and 400 mM of MSG was used to evaluate the GABA yield of *L. brevis.* In this case, the best result was obtained at 270 mM (Villegas et al., 2016).

Despite the efficacy of direct addition of Glu or MSG, alternatives have been sought in order to reduce economic costs (Xu et al., 2017). For example, Woraharn et al. (2016) employed the mushroom *Hericium erinaceus* as a source of Glu coupled with the co-culture of two *Lactobacillus* strains. *Lactobacillus brevis* was used to hydrolyse the L-glutamine to Glu using an L-glutaminase, and *Lactobacillus fermentum* was added to transform this Glu into GABA. Another technique to promote Glu secretion without external supplementation is co-cultivation with a microorganism that synthesises Glu. Yang et al. (2015) used a *Corynebacterium glutamicum* strain to produce Glu, which was then transformed into GABA by *Lactobacillus plantarum* through fermentation of cassava powder.

Furthermore, the addition of different carbon and nitrogen sources can help improve bacterial metabolism and therefore enhance GABA synthesis. Zareian et al. (2012) used glucose and nitrogen to enhance bacterial production of Glu without external supplementation. Afterwards, the process was adjusted to increase Glu and GABA production by three- and ten-fold, respectively (Zareian, Ebrahimpour, Sabo Mohamed, & Saari, 2013).

Lim, Cha, Roh, Shin, and Seo (2017) used different carbon and nitrogen sources to analyse variations in GABA production by *Lactobacillus brevis* and found that maltose and tryptone in presence of MSG yielded a major increase in GABA production. However, the optimal carbon and nitrogen source depends on the *Lactobacillus* strain. Several studies have shown that glucose is the most effective carbon source for *Lactobacillus plantarum* (Chen, Xu, & Zheng, 2015) and *Lactobacillus brevis* (Hasegawa et al., 2018). Likewise, Zhao et al. (2015) have reported that *Lactobacillus buchneri* produces a higher amount of GABA in the presence of xylose. Yi Song and Yu Chui (2017) observed that *Lactobacillus rhamnosus* synthesises a high amount of this amino acid using galactose.

Regarding the nitrogen source, Binh, Ju, Jung, and Park (2014) observed an increase in GABA synthesis by *Lactobacillus brevis* in the presence of 2% casein peptone or yeast extract. Saraphanchotiwitthaya and Sripalakit (2018) also analysed how *Lactobacillus brevis* behaves with different nitrogen sources, obtaining the best results with 1% peptone.

Other procedures can also be used to enhance GAD activity, such as coenzyme PLP supplementation (Shan et al., 2015), regulation of Tween-80 concentration (Wang et al., 2018) and the addition of metal ions (Lin et al., 2017).

GABA yield is also influenced by culture media and their nutrient concentration. Most studies have used MRS broth (Man, Rogosa & Sharpe) supplemented with Glu or MSG. This broth is suitable to optimise GABA production due to its high concentration of nutrients (Chen et al., 2015; Cho, Park, Kim, Ryu, & Park, 2011). Some researchers have explored more natural media, such as grape must, dairy products (Di Cagno et al., 2010), barley grains and kidney beans (Saraphanchotiwitthaya & Sripalakit, 2018), with a view to industrial application.

2.3.3. Effect of cultivation time

The point at which optimum GABA production is reached varies depending on the *Lactobacillus* strain employed. For example, Tajabadi et al. (2015) detected the highest GABA yield after 60 h of cultivation using *Lactobacillus plantarum*, whereas Shan et al. (2015) reported a higher amount of GABA at 35 h using another strain of *Lactobacillus plantarum.* Similar results were obtained in a study of *Lactobacillus brevis*, where the highest amount of GABA was reached at 30 h (Lim et al., 2017).

3. Beneficial effects of GABA and probiotics on human health

In recent years, many researchers have focused on the effect of a group of molecules produced by different bacteria as a result of metabolism called postbiotics, which can help protect against human diseases such as diabetes, cardiovascular diseases and brain disorders.

Despite affecting different organs, these postbiotics act mainly via the brain-gut connection (Bienenstock, Forsythe, Karimi, & Kunze, 2010). The scaffolding of the gut-brain axis includes the GI tract, CNS, autonomic nervous system, enteric nervous system, neuroendocrine system and immune system (Kraimi et al., 2019).

GABA is an important postbiotic considered an inhibitory neurotransmitter that has aroused increasing interest over the years due to its essential role in the nervous system (Sherwin, Sandhu, Dinan, & Cryan, 2016). The inhibitory effect of GABA occurs as a result of binding to the GABAergic receptor system composed of three specific receptors: $GABA_A$, $GABA_B$ and $GABA_C$ (Rissman & Mobley, 2011). Through these receptors, GABA can modulate mood (e.g. relaxation), sleep disorders and temporal and spatial memory (Sigel & Steinmann, 2012). Beneficial effects have also been demonstrated in epilepsy (Bagheri, Heydari, Alinaghipour, & Salami, 2019), depression (Boonstra et al., 2015), diabetes (Abdelazez et al., 2018), asthmatic disorders (Forkuo et al., 2017) and cancer (Song et al., 2016; Wang et al., 2016). Moreover, several studies have demonstrated the importance of GABA in the development of neural diseases such as schizophrenia (Turkheimer, Leech, Expert, Lord, & Vernon, 2015), Alzheimer's disease (Mele, Costa, & Duarte, 2019), Parkinson's disease (Cassani et al., 2015) and Huntington's disease (Hsu, Chang, & Chern, 2018; Ogawa et al., 2018), as will be discussed below.

3.1. Cardiovascular disorders

GABA has many physiological effects on human health, the most important of which is its cardiovascular effect. The WHO has described hypertension as one of the key risk factors for the development of cardiovascular disease, affecting one billion people worldwide. Hypertension usually leads to heart attacks and strokes, killing millions of people every year (WHO, 2013).

The hypotensive mechanism of GABA is based on the inhibition of noradrenaline released from the peripheral sympathetic nerve terminals that inhibit perivascular nerve stimulation (Nejati et al., 2013). Kimura, Hayakawa, and Sansawa (2002) evaluated the hypotensive effect of GABA compound by injecting this neurotransmitter into the duodenum. Their results showed that a minimal amount of GABA could reduce blood pressure through activation of $GABA_B$ receptor. Subsequently, Inoue et al. (2003) developed a fermented milk enriched with *Lactobacillus casei*, a known Glu producer, and *Lactococcus lactis*, which synthesises GABA. The beverage was first tested in spontaneously hypertensive rats, and intake was associated with a reduction in blood pressure. The effect of this fermented milk was then tested on patients with mild hypertension, and the results indicated that a daily intake of 20 mg of GABA reduced their blood pressure. More recently, Abd El-Fattah, Sakr, El-Dieb, and Elkashef (2018) have also demonstrated the antihypertensive effects of GABA. They applied different treatments to milk enriched with probiotics such as *L. helveticus* or *L. rhamnosus*, which produce GABA, for subsequent production of a functional yoghurt. The beneficial effects of the yoghurt, rich in bioactive compounds including GABA, were evaluated by measuring angiotensin-converting enzyme inhibitory activity, thrombin inhibition activity, inhibition of cholesterol micellar solubility and antioxidant activity, all of which are related to cardiovascular health.

Other studies have focused on the production of GABA-enriched foods without the addition of LAB. Germinated brown rice is one promising example as it contains several bioactive compounds, including GABA, and shows beneficial health effects such as antihyperlipidemic and antihypertensive actions, which could help reduce the risk of developing cardiovascular disease (Wu, Yang, Touré, Jin, & Xu, 2013). In light of these potential benefits, Cáceres, Peñas, Martinez-Villaluenga, Amigo, and Frias (2017) have developed a germinated brown rice variety which could increase GABA intake.

3.2. Nervous system disorders

3.2.1. Epilepsy

Epilepsy is considered a major public concern because it is a chronic neurological disorder affecting more than 50 million people worldwide that is characterised by seizures (Lum, Olson, & Hsiao, 2019). Although the different mechanisms that provoke seizures have not yet been fully clarified, it seems that alterations in the ion transport functionality, synaptic connectivity and neurotransmitter activity of Glu and GABA may be involved in an imbalance in CNS modulation (Dahlin & Prast-Nielsen, 2019). DeLorey and Olsen (1999) have reported a relationship between the development of epilepsy and alterations in the GABA network, describing how disruption of PLP metabolism in rats was associated with a reduction in GABA concentration and subsequent spontaneous seizures in these animals.

Generally, anticonvulsant drugs increase GABA availability or enhance GABA-mediated inhibition (Pfeiffer, Draguhn, Meierkord, & Heinemann, 1996). Abou-Khalil (2019) has summarised the most common anticonvulsant drugs currently in use, which include medications that stimulate the $GABA_A$ receptor, such as phenobarbital and benzodiazepines, and others that modulate GABA concentration, such as felbamate, valproate and gabapentin. All these drugs entail unavoidable side effects and drug resistance.

Several studies have associated epilepsy with alterations in gut-brain axis functionally produced by dysbiosis (Dahlin & Prast-Nielsen, 2019). Bagheri et al. (2019) have reported that supplementation with the probiotics *L. rhamnosus*, *L. reuteri* and *B. infantis* reduced seizure severity in animal models, increased GABA activity and improved oxidative balance. Further clinical analyses are required to confirm the therapeutic effect of these probiotics.

3.2.2. Anxiety and depression

The worldwide prevalence of mental disorders such as depression or anxiety has increased dramatically in recent decades.

Depressive disorders are characterised by sadness, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, feelings of tiredness and poor concentration. Depression can be chronic or episodic, substantially impairing an individual's ability to function at work or school or cope with daily life (WHO, 2017).

Anxiety disorders refer to a group of mental disorders characterised by feelings of anxiety and fear, and include generalised anxiety disorder, panic disorder, phobias, social anxiety disorder, obsessive-compulsive disorder (OCD) and post-traumatic stress disorder (PTSD). As with depression, symptoms can range from mild to severe. The duration of symptoms typically experienced by people with anxiety disorders renders these chronic rather than episodic disorders (WHO, 2017).

Depression and anxiety can be triggered by the disruption of various physiological pathways. Previously, depression research had focused on the alteration of monoamine production; however, current studies have highlighted the influence of neuro-endocrinological abnormalities and alteration of the Glu/GABA system, among others. Although the pathophysiology of anxiety is unclear, studies have also demonstrated the influence of alteration in the Glu/GABA system (Saki, Bahmani, & Rafieian-Kopaei, 2014). For instance, Lacerda-Pinheiro et al. (2014) have confirmed that GABA is highly involved in anxiety processes since $GABA_A$ receptor is the active site of anxiolytic drugs. Meanwhile, Luscher, Shen, and Sahir (2011) have presented evidence that supports the important role of GABA concentration and GABA receptor functionality in the development of depression and anxiety.

Anxiety and depressive disorders can be treated with antidepressant drugs that modulate monoaminergic neurotransmission, GABAergic transmission or GABA receptors (Möhler, 2012). Soussan and Kjellgren (2016) have postulated that GABA has better effects and creates less dependence.

In contrast, Foster and McVey Neufeld (2013) have reported a connection between gut-brain axis disruption and risk of anxiety and depression. Several studies have assessed the effectiveness of probiotics in alleviating anxiety and depression. Bravo et al. (2011) have demonstrated that the expression of GABA receptors linked to behavioural aspects of anxiety is modulated in rats by the administration of *Lactobacillus rhamnosus*. In addition, Boonstra et al. (2015) have reported that mice fed with bacteria from the genus *Lactobacillus* showed

behaviour differences, displaying antidepressant-like behaviours and being less anxious than controls. Clinical trials have also been conducted to evaluate probiotic performance. Messaoudi et al. (2011) have confirmed the efficacy of *Lactobacillus helveticus* and *Bifidobacterium longum* in depressive patients. Subsequently, Strandwitz et al. (2019) showed that the probiotics *Lactobacillus* and *Bifidobacterium* were producers of the GABA molecule, supporting the vast potential of these microorganisms in treating anxiety and depression disorders.

3.2.3. Drug addiction

Stress and depressive disorders are among the most common reasons for developing a drug addiction. In addition, preclinical and clinical studies have suggested a connection between microbiome perturbation and the risk of addiction (Skosnik & Cortes-Briones, 2016), while Cao, Shi, Hao, Wu, and Li (2016) have elucidated the key role of the GABA system in the development of drug addiction. Karila et al. (2010) have described the potentially beneficial effects on methamphetamine dependence of administering GABA agonists such as gabapentin or vigabatrin. In another clinical trial, Kampman et al. (2004) assessed the influence of topiramate, a GABA enhancer that increases the concentration of this molecule in the brain, in people with cocaine addiction. Their results suggest that this drug might help promote abstinence. Lastly, Filip et al. (2015) have described a new drug abuse therapy model based on GABA receptor regulation. However, we found no studies that had used probiotic GABA producers to treat drug addiction.

3.2.4. Neural diseases

GABA concentrations and the correct behaviour of GABA receptors play an essential role in the development of many kinds of neural disease. Several studies have indicated that some kinds of brain injury are related to abnormalities in the patient's neurotransmission. For example, hypoxic-ischaemic events during foetal development can trigger learning and memory deficits due to neurotransmission disruption produced by permanent damage in GABA function (Cunha-Rodrigues, Balduci, Tenório, & Barradas, 2018). Akhoundzadeh, Abedin, Shadnoush, and Sadeghzadeh (2018) performed an experiment in mice to determine whether consumption of *Lactobacillus* and *Bifidobacterium* strains, which can modulate GABA and serotonin concentrations, exerted a beneficial effect on brain ischaemia. They obtained promising results, paving the way for use of this kind of probiotic to prevent or attenuate cerebral stroke injury.

Other neural diseases, including schizophrenia, Alzheimer's, Parkinson's and Huntington's disease, have been linked to dysbiosis due to the strong connection between gut and brain. Given the important role that the gut microbiota plays in human health, researchers have also focused on the connection between ageing-related changes in the microbiota and neural diseases, which have a higher incidence in older people. Goldman and Postuma (2014) have described the role of microbiota disorders in the early stages of Parkinson's disease, and their results have been confirmed by Cassani et al. (2015), who reported the important role of gut dysbiosis from the early stages of Parkinson's disease.

It has been shown that all of these diseases can be affected by GABA, among other factors. Turkheimer et al. (2015) have provided evidence relating GABA disorders and schizophrenia. Moreover, GABAergic dysfunction has been observed in Huntington's disease (Ogawa et al., 2018). An imbalance in the glutamatergic system plays an important role in motor and behaviour control dysfunction, clear symptoms of the disease (Hsu et al., 2018).

Although there is still no treatment for Huntington's disease, several approaches have been explored to improve symptoms in affected patients. Recent studies have tried to obtain a better understanding of the disease, observing a lower GABA concentration in affected patients (Boonstra et al., 2015). As regards Alzheimer's disease, Seidl, Cairns, Singewald, Kaehler, and Lubec (2001) found a significant loss of GABA in different regions of the brain (temporal and occipital cortex, as well as in cerebellum) in affected patients. The expression of GABA transporters seems to be involved in the progression of the disease (Fuhrer et al., 2017), and an imbalance in the GABAergic system has also been related to Alzheimer's disease (Mele et al., 2019).

3.3. Diabetes

Diabetes is characterised by dysfunctional pancreatic cells that no longer produce insulin, affecting glucose levels. The incidence of diabetes worldwide has risen dramatically, and it is now considered one of the main threats to human health.

The therapeutic effect of GABA against diabetes has been widely studied. Tian et al. (2014) have reported that the inflammatory response and the progression of pre-diabetes can be inhibited by administering the GABA molecule as a therapeutic agent. Wang, Prud'homme, and Wan (2015) have demonstrated the regulatory effect of the GABA molecule on human islets involved in diabetes, highlighting the suppression of insulitis and systemic inflammatory cytokine production.

Abdelazez et al. (2018) have recommended pharmaceutical and food applications of GABA produced by their LAB strains, as it has shown a clear effect in reducing glucose and insulin levels in plasma in *in vivo* experiments, and could therefore be used to reduce the incidence of type 1 diabetes mellitus.

3.4. Cancer

Cancer is one of the main causes of death worldwide and is characterised by the rapid creation and proliferation of abnormal cells, which can affect different organs of the body.

Many studies have shown that the GABAergic pathway is altered in cancer patients. Brzozowska, Burdan, Duma, Solski, and Mazurkiewicz (2017) have demonstrated that GABA has significant prognostic value in breast cancer. According to their results, higher amounts of GABA in patients were related to a better survival prognosis.

Most studies have confirmed that tumour cell proliferation can be suppressed by activating GABA receptors, which are expressed in some brain structures and in many organs, where they are responsible for neuronal stimulation and hormonal secretion. Shu et al. (2016) have reported that activation of metabotropic GABA receptor signalling significantly inhibits the colorectal tumour cell HT29. According to the results reported by Wang et al. (2016), the GABA molecule inhibits the growth of a cholangiocarcinoma cell line. In addition, Song et al. (2016) found that the GABA molecule inhibited proliferation of colon cancer cells, suggesting the use of GABA in polychemotherapy of colon cancer.

3.5. Asthma

GABA receptors in the CNS are distributed throughout the body, including the lungs. These receptors appear to be dysfunctional in patients with asthma, inhibiting the contraction of airway smooth muscle (Dicpinigaitis, 1999). Yocum et al. (2017) have demonstrated that knock-out of a specific GABA receptor worsens the symptoms of allergic asthma, increasing the inflammatory response and airway reactivity.

Arnold et al. (2016) have targeted GABA receptors in the lungs as an approach for asthma treatment, while Forkuo et al. (2017) have reported a reduction in airway hyperresponsiveness when new ligands, considered possible novel oral drugs, were used as GABA receptor modulators for asthma treatment.

All these studies demonstrate the potential of the postbiotic GABA as a bioactive compound to help prevent and treat highly prevalent diseases in today's society. Below, Table 3 gives a detailed list of the disorders in which GABA is involved and the beneficial effects that it can exert.

Table 3
The association between gamma-aminobutyric acid, probiotics and human diseases.

Health disorder	Disease	GABA effect	Reference
Cardiovascular disorder triggered by hypertension	Heart attack and stroke	Hypotensive effect	Abd El-Fattah et al. (2018) Cáceres et al. (2017), Inoue et al. (2003), Nejati et al. (2013)
Alteration of nervous system functionality	Huntington's disease	Inhibits neurotransmission	Boonstra et al. (2015), Hsu et al. (2018)
	Alzheimer's disease	Inhibits neurotransmission	Seidl et al. (2001), Fuhrer et al. (2017), Mele et al. (2019)
	Drug abuse therapy	Inhibits neurotransmission	Filip et al. (2015), Cao et al. (2016)
	Learning and memory disorders	Enhances temporal and spatial memory	Cunha-Rodrigues, Balduci, Tenório, and Barradas (2018)
	Anxiety and depression	Relaxant and antidepressant effect	Boonstra et al. (2015), Bravo et al. (2011), Soussan and Kjellgren (2016)
	Epilepsy	Reduce seizure severity	Bagheri et al. (2019)
Metabolic disorders of carbohydrate metabolism	Diabetes type I	α-cells: GABA induces membrane hyperpolarization and inhibits glucagon secretion. β-cells: GABA induces membrane depolarization and enhances insulin secretion.	Wang et al. (2015), Tian et al. (2014)
Uncontrolled growth and spread of cells.	Cancer	Delays and/or inhibits cancer cell proliferation Stimulatory action on cancer cell apoptosis Potent tumour suppressor	Brzozowska et al. (2017), Song et al. (2016), Shu et al. (2016), Wang et al. (2016)
Airway inflammation	Asthma	Control in asthma Enhances immunity	Arnold et al. (2016), Yocum et al. (2017), Forkuo et al. (2017)

4. Present and future of GABA and probiotics

As has been seen, the gut microbiota plays a very important role in various body functions, and according to recent studies, its imbalance, known as dysbiosis, is related to a range of health problems from Crohn's disease to cancer. Thanks to advances in high-throughput sequencing and metabolomics, it has been shown that certain compounds called postbiotics can change microbiota function and even play a very important role in disease prevention (Barrett, Ross, O'Toole, Fitzgerald, & Stanton, 2012).

One of these postbiotics is GABA, which has attracted increasing interest in recent years due to its wide variety of potential health benefits such as blood pressure control, but especially due to its role as a neurotransmitter and its use in the treatment of anxiety and depression. This compound can be obtained from several microorganisms and plants, but its concentration in these matrices is low and the process is expensive. As an alternative, it can be obtained via chemical synthesis, but this process requires the use of corrosive reagents. Consequently, the use of probiotics as a more sustainable route for postbiotic production is gaining interest among the scientific community and industrial sector, specifically from LAB. Today, the objectives of GABA production are focused on seeking highly productive GABA strains and optimising the growth conditions for these bacteria (Diana, Quílez, & Rafecas, 2014). The food industry is mainly interested in GABA production because it is considered a bioactive compound that promotes health and is useful for the Development of Foods for Specified Health Use (FOSHU) (Martirosyan & Singh, 2015). For instance, Cáceres et al. (2017) and Cho and Lim (2016) have improved GABA content in brown rice by means of a germination process, while Abd El-Fattah et al. (2018) have developed a functional yoghurt rich in bioactive compounds, including GABA.

Another aspect on which current research on the use of GABA for health applications —and especially brain health— now focuses is to clarify whether GABA peripherally generated by probiotic bacteria is capable of crossing the blood-brain barrier and affecting GABAergic neurotransmission (Boonstra et al., 2015). Studies in animals have shown that the gut microbiota can regulate GABAergic neurotransmission across the vagus nerve (Bravo et al., 2011; Janik et al., 2016), which is the main route from the abdominal cavity to the brain. The gut microbiota can activate this route to mediate effects on the brain.

Preliminary clinical trials indicate the potential of GABA-producing probiotics in the treatment of neuropsychiatric conditions such as depression (Singh et al., 2018). However, greater investment by food and nutraceutical manufacturers in research at industrial scale is required in order to run larger-scale clinical trials and verify their effectiveness under certain conditions (Dinan & Cryan, 2016).

It is necessary to determine the gut metabolites' synergistic and antagonistic effects with postbiotics such as the GABA. In addition, there is a need to study the clinical effects of these postbiotics in healthy individuals and sick people alike through the application of nutrigenomic approaches.

Advances in "omics" technologies and culture-independent techniques have led to significant progress in the quest to identify bacteria that produce postbiotics which influence the host's physiology and immune function. The application of computer tools has yielded greater knowledge about the mechanisms of bioactive compounds and their correlation with the intestinal microbiota, while use of high-throughput sequencing from metagenomic and meta-transcriptomic sequencing (from cDNA libraries) has revealed the relationship between probiotics and the host's gut microbiome and will no doubt identify new postbiotic-producing bacteria for future use in the treatment of highly prevalent diseases.

In addition, the use of technologies such as fluorescence *in situ* hybridisation combined with single-cell imaging, metabolic oligosaccharide engineering and bio-orthogonal click chemistry has enabled *in vivo* monitoring of microbial populations. All of this will contribute to advances in knowledge of the effect of postbiotics on host physiology, enabling the development of personalised therapies (Singh et al., 2018).

Currently, the development of probiotics, prebiotics and postbiotics has several limitations, especially related to a lack of knowledge about the intestinal flora in disease or homeostasis (Klemashevich et al., 2014). Another factor that limits the application of probiotics in the treatment of various diseases is their mechanism of action, since multiple routes may be involved in their health benefits and the effectiveness of probiotics can vary from one person to another depending on their intestinal microbiota. However, there is substantial evidence of their beneficial effect on health and their potential to treat various diseases (Sherwin et al., 2016). Thus, increasing scientific evidence supports the important role of the gut microbiota in health, disease prevention and even treatment, and it is highly probable that therapies and treatments will be developed based on functional foods and supplements containing probiotics, prebiotics and postbiotics to combat highly prevalent diseases in an ageing population, from cardiovascular

Table 4
Global market forecast for probiotics in several applications.

Application	2016	2017	2022	Compound annual growth rate (CAGR%) 2017–2022
Food and beverages	26,647.3	28,580.5	41,882.1	7.9
Dietary supplements	6,469.9	6,954.2	10,012.7	7.6
Animal feed	3,436.0	3,697.8	5,320.9	7.5
Total	36,553.2	39,232.5	57,215.7	7.8

disease to cancer and neuropsychiatric disorders.

However, there is a need for adequate legislation to regulate key aspects related to probiotics, including efficacy, safety and quality control in product manufacture, and to regulate the health claims that can be made for individual products.

One of the main issues is the lack of international regulation, which generates uncertainty among food and health professionals. In the European Union, products with probiotics are regulated by the Food Products Directive and Regulation (regulation 178/2002/EC, Directive 2000/13/EU). The European Food Safety Authority (EFSA) is responsible for authorising such health claims in accordance with Regulation 1924/2006. As this institution considers that the characterisation of probiotics is insufficient, all the health claims attributed to probiotics have been rejected. In the USA, the Food and Drug Administration (FDA) does not support probiotic health claims either; however, it has recognised that they may reduce the likelihood of developing a disease. Both the EFSA and the FDA agree on the need for more clinical trials and more personalised studies of the effects of probiotics in healthy and sick people (Donovan, Schneeman, Gibson, & Sanders, 2012).

In both cases, the current regulations do not take into account the complex nature of probiotic products or that their properties depend on the species, strains and manufacturing procedures employed. Regulation is necessary when probiotics are used to treat pathologies, so as to determine the effect on health of specific formulations through scientific studies (de Simone, 2019). Some have suggested that probiotic products with an effect in the prevention or treatment of certain diseases should be considered adjuvant drugs rather than dietary supplements and should comply with rigorous legislation, due to the possible harmful effects of their use depending on the microbiota of the individual (Kothari, Patel, & Kim, 2019).

4.1. Probiotic market development

The prevalence of diseases such as cardiovascular disease, depression and cancer is rising as a result of population ageing. Therefore, probiotics and postbiotics such as GABA have attracted increasing interest because of their effects on health. Despite the current limitations indicated earlier, the growing importance of new technologies and progress in research on the effect of probiotics and postbiotics on human health based on the microbiota will undoubtedly play a key role in the development of personalised therapies for highly prevalent diseases.

Probiotics are primarily used in the food and beverage industry, which accounts for almost 72.9% of the market share, followed by the dietary supplements and animal feed industries, accounting for approximately 17.7% and 9.4%, respectively, according to market research carried out by BBC Research in 2018 (Karthik, 2018).

This study reported that the global market for probiotics accounted for almost 36.6 billion dollars in 2016 and is expected to reach 57.2 billion dollars in 2022, with an annual growth rate of 7.8% from 2017 to 2022 (Table 4). This market is driven by the food and beverage industry, which is expected to maintain its leading position in this period. The Asia-Pacific region is expected to be the fastest growing area (with 8.1% annual growth). The most commonly used probiotic is *Lactobacillus*, accounting for almost 63.1% of the market share, followed by *Bifidobacterium* and *Streptococcus*, accounting for 27.6% and 4.2%, respectively.

Within probiotics, products based on GABA-generating probiotics are going to be in high demand because of the beneficial nature of this compound for the maintenance of brain health. The global market for nutraceutical products produced to improve memory and brain health, in which products with GABA or products which promote the generation of GABA could be included, was estimated at 4.3 billion dollars in 2017 and is expected to reach 6.7 billion by 2023, growing at a compound annual rate of 7.8% from 2018 to 2023. In 2017, this market was dominated by North America and the Asia-Pacific region, with an estimated market share of 32.6% and 30.2%, respectively. The North American market was estimated at 1.4 billion dollars in 2017 and is expected to reach 2.2 billion dollars by 2023, growing at a compound annual growth rate (CAGR) of 9.5% from 2018 to 2023. The market in the Asia-Pacific region was estimated at 1.3 billion dollars in 2017 and is expected to reach 2.1 billion dollars by 2023, growing at a CAGR of 8.4% from 2018 to 2023. The European market was estimated at 1.2 billion dollars in 2017 and is expected to reach 1.8 billion dollars by 2023, growing at a compound annual rate of 6.7% from 2018 to 2023 (Agheyisi, 2014; Karthik, 2018).

Consequently, probiotic foods and supplements in general and GABA producers in particular are in high demand. Once their effect on health has been scientifically validated, it is expected that they will form part of a personalised diet/therapy for the prevention and treatment of highly prevalent diseases.

5. Conclusions

Probiotic's health effects have been under research for a long time and many studies have proved its benefits through *in vitro* and *in vivo* experiments. Currently, investigations are based on the potential of the metabolites produced by probiotics, known as postbiotics, and how the combination of probiotics and postbiotic, could have beneficial effects in humans. Among postbiotics, is important to highlight the importance of GABA in health, since the imbalance of this neurotransmitter has been related to diseases of different aetiology. Therefore, the development of food products enriched with probiotics and GABA, that could prevent and relief the symptomatology of those diseases, is expected to increase in the future.

Ethics statement

The authors declare no ethical issue related with this article.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Characterisation of the probiotic potential of *Lactiplantibacillus plantarum K16* and its ability to produce the postbiotic metabolite γ-aminobutyric acid

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ABSTRACT

Lactiplantibacillus plantarum has been widely studied due to its beneficial effects on health such as protect against pathogens, enhance the immune system, or produce metabolites like γ-aminobutyric acid (GABA). The objective of this study was the evaluation of the GABA-producer *L. plantarum K16* isolated from kimchi. The safety and probiotic characterisation of this strain was performed by analysing carbohydrates fermentation, enzymatic activity, antibiotics susceptibility, and haemolytic and antimicrobial activity. Likewise, GABA production was optimised following a one-factor-at-a-time procedure by changing relevant fermentation parameters like incubation temperature, yeast extract concentration and fermentation time. The results indicated that *L. plantarum K16* has the potential to stimulate the digestion and absorption of several nutrients and it could have an inhibitory effect against pathogenic bacteria. The best results for GABA production by this strain was around 1000 mg/L, using 12 g/L of yeast extract, 34 °C of incubation temperature and 96 h of fermentation time.

1. Introduction

Fermented foods and beverages have been broadly used for the last centuries due to their high nutritional and potential therapeutic effects produced by the wide variety of probiotic microorganisms contained in these foods (Ozen & Dinleyici, 2015). The International Scientific Association for Probiotics and Prebiotics (ISAPP) ratified the Food and Agriculture Organization definition (2002) of probiotics claiming that they are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014; Chávarri et al., 2010). Generally, fermented dairy products have been known as the primary source of probiotic microorganisms (Zucko et al., 2020). However, the increased demand of industry and costumers for these beneficial microorganisms has expanded the research area to non-dairy fermented products based on vegetables, legumes, cereals, or fish, such as Ngari, Tempeh, Sauerkraut, Kimchi or Boza (Ilango & Antony, 2021). Several well-known probiotics such as *Bacillus* (Park et al., 2021a), *Lactobacillus* (Pérez-Díaz, Johanningsmeier, Anekella, Pagán-Medina, Méndez-Sandoval, Arellano, Price, & Daughtry, 2021), *Enterococcus* (Baccouri, Boukerb, & Farhat, 2019), *Aspergillus oryzae* (Park, Seo, & Kim, 2019), *Bifidobacterium* (Yasmin et al., 2020), and *Saccharomyces cerevisiae* (Syal & Vohra, 2013) have been widely isolated from these types of traditional fermented foods.

Furthermore, there is a need to assess the safety and effectiveness of these microorganisms through different types of *in vitro* studies to consider them as generally regarded as safe (GRAS) and, thus, classify them as probiotics. For that purpose, several researchers have evaluated the ability of these microorganisms to produce hazardous compounds, survive against stressful environments, protect against pathogens, or synthesise beneficial products (Chavarri, Diez-Gutiérrez, Marañón, Villarán, & Barron, 2022). For instance, Son et al. (2018) assessed the probiotic activity of lactic acid bacteria (LAB) isolated from traditional Korean fermented foods by analysing enzymatic activity, adhesion capacity to intestinal cells, antibiotic resistance, or the ability to synthesise

Abbreviations: EFSA, European Food Safety Authority; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GRAS, generally regarded as safe; ISAPP, International Scientific Association for Probiotics and Prebiotics; LAB, lactic acid bacteria; L-Glu, L-glutamate; MRS, Man Rogosa Sharpe; MSG, monosodium glutamate; OFAT, one-factor-at-a-time.

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β-glucosidase. In addition, Kumari, Angmo, and Monika (2016) determined the biochemical profile of *Lactobacillus* isolated from fermented foods traditionally made in the Himalayas, and they evaluated the ability of these bacteria to go through biological barriers, haemolytic activity, and cell-surface interactions.

The characterisation of probiotics has made it possible to find a wide variety of microorganisms that can enhance human health, such as reinforcing the host's immune system, protecting against pathogen colonisation, and stimulating the release of bioactive compounds. Among the well-known probiotics, *Lactobacillus plantarum* has been extensively studied due to its potential beneficial effects on human health. Recently, Zheng et al. (2020) performed a depth phylogenetic study that changed the classification of the genus *Lactobacillus* and, thus, *Lactobacillus plantarum* was newly classified as *Lactiplantibacillus plantarum*. *L. plantarum* is a facultative anaerobe heterofermentative microorganism included in the Group *B Lactobacillus* classification, mainly isolated from vegetables-based food products (Todorov & de Melo Franco, 2010). Mao et al. (2021) analysed several *L. plantarum* strains isolated from different food matrices. They reported that according to the isolated source, the metabolism of each strain could be different, highlighting that protein and lipid metabolism is highly conserved. However, the carbohydrates consumption and amino acid catabolism could present a significant variation. Hence, the yield variability of the primary metabolism of *L. plantarum* could substantially impact other metabolic pathways involved in the production of bioactive compounds, known as postbiotics, which could have several beneficial effects on human health (Peluzio, 2021). Studies have recently indicated that the postbiotic term includes the metabolites produced or other compounds released by probiotics during fermentation (Abdelazez et al., 2022; Kim, Lee, Kim, Kim, and Yoon (2022a).

Regarding the postbiotic metabolites, different organic compounds could be found in this classification, such as vitamins, amino acids, proteins, short-chain fatty acids or neurotransmitters, characterised according to their main function in human health (Mojgani & Dadar, 2021). For instance, it has been reported that the production of short-chain fatty acids from the metabolism of galactooligosaccharides improves the immune system promotes cell differentiation or maintains the intestinal microbiota (Fuhren et al., 2020; Tran et al., 2020). Moreover, the metabolism of amino acids, such as aspartic acid or tryptophan, could lead to the synthesis of essential human compounds, including hormones, nucleic acids or neurotransmitters (Chávarri, Diez-Gutiérrez, Marañón, & Barron, 2021).

Among postbiotic metabolites, GABA is a non-protein amino acid extensively produced by LAB, such as *L. brevis* (Liu, Li, Liu, Ko, & Kim, 2022), *L. plantarum* (Kim et al., 2022b), *L. rhamnosus* (Song & Yu, 2018) or *L. lactis* (Sharma et al., 2022). The synthesis of this postbiotic compound depends on the amino acid L-glutamate (L-Glu) because it is used as a precursor of the glutamic acid decarboxylase (GAD) biosynthetic pathway (Falah, Vasiee, Tabatabaei-Yazdi, Moradi, & Sabahi, 2022). Likewise, the production process is closely related to specific fermentation parameters, including incubation temperature, concentration of carbon and nitrogen sources, type and concentration of minerals and fermentation time (Dahiya & Manuel, 2021). Recently, GABA has gained importance due to its ability to improve human health through the modulation of blood pressure, protection against nervous system disorders, preventing metabolic diseases such as diabetes, and reducing pro-inflammatory cascades (Diez-Gutiérrez, San Vicente, & Barrón, 2020). For example, Yunes, Poluektova, and Vasileva (2020) reported the antidepressant effect in mice produced by *L. plantarum 90sk* combined with *B. adolescentis 150* strains, which presented high production of GABA. Zareian, Oskoueian, Forghani, and Ebrahimi (2015) investigated the blood pressure modulation and the antioxidant effect of GABA by feeding hypertensive rats with a GABA-enriched fermented beverage. The results of this study showed that the consumption of GABA enhanced the modulation of norepinephrine and triggered the overexpression of the endothelin-1 protein, which is one of the most relevant factors affecting the hypertension modulation. These wide benefits of GABA and probiotic microorganisms, like *L. plantarum* strains, have opened a new possibility to address the demand of new functional ingredients (Zhang et al., 2022a; Jin et al., 2022). Considering the above-mentioned background, the objective of the present study was the characterisation of the probiotic ability and safety of *L. plantarum K16* strain isolated from Kimchi. Additionally, the effect of incubation temperature, nitrogen source (yeast extract concentration) and fermentation time on the production of GABA in Man Rogosa Sharpe (MRS) by *L. plantarum K16* strain was studied through a one-factor-at-a-time (OFAT) experimental design. The results of these experiment will give the information to know if *L. plantarum K16* and the amount of GABA produced are good enough to use them as potential functional ingredients.

2. Materials and methods

2.1. Isolation and identification of L. plantarum K16 strain

LABs were isolated from Kimchi using a standard culturing method described by Monika, Kumar, Kumari, Angmo, and Bhalla (2017). The ability of LABs to produce GABA was assessed by growing them in MRS broth (Sigma-Aldrich, Madrid, Spain) supplemented with 1 % of L-Glu (Scharlab, Barcelona, Spain) at 37 °C for 48 h and the supernatants obtained were analysed with ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS). The only LAB strain that seemed to produce GABA was finally sequenced and identified as *L. plantarum K16*.

2.2. Safety and probiotic characterisation of L. plantarum K16 strain

The characterisation of *L. plantarum K16* was performed focusing on the analysis of the biochemical profiling of the strain through the analysis of the metabolism of carbohydrates and its enzymatic activity, as well as its potential to inhibit the growth of pathogens. Furthermore, the safety of the strain was evaluated carrying out the haemolytic test and the susceptibility of *L. plantarum K16* strain to several antibiotics (Angmo, Kumari, & Savitri, 2016; Dowarah, Verma, Agarwal, Singh, & Singh, 2018).

2.2.1. Carbohydrates metabolism

L. plantarum K16 strain was grown for 24 h in MRS agar plates at 37 °C, and 5 % of high purity carbon dioxide (Nippon Gases, Madrid, Spain). Afterwards, the profiling of carbohydrates fermentation was analysed using the Analytical Profile Index (API) 50 CHL kit (APISystem, La Balme les Grottes, France), which is based on 50-wells of different fermentable carbohydrates. According to the procedure described by Salleh, Lani, Chilek, Kamaruding, and Ismail (2021), the strain was inoculated into the wells and the strips were incubated for 48 h at 37 °C. The API and the API web (https://apiweb.biomerieux.com) were used to evaluate the results on carbohydrates metabolism.

2.2.2. Enzymatic profiling

Enzymatic activity of *L. plantarum K16* was determined using API ZYM kit (APISystem) which was used to test the activity of 19 different enzymes. The inoculated strips were incubated at 37 °C for 4 h and, after addition of ZYM A and B reactive, the enzymatic activity of the strain was determined by colour intensity and were expressed as nmol of substrate hydrolysed according to previously described (Stoyanovski et al., 2013).

2.2.3. Antibiotic susceptibility

Disk-diffusion antibiotic susceptibility test was used to evaluate the antibiotics resistance of *L. plantarum K16* (Dowarah et al., 2018). The strain was grown overnight, spread on MRS agar plates, and incubated for 48 h at 37 °C. The length of the diameter of the inhibition zone was

measured in millimetres (±0.1) for all antibiotics and, according to the size, the bacteria was considered susceptible (≥21 mm), intermediate (16–20 mm) or resistance (≤15 mm) to the antibiotic.

2.2.4. Haemolytic activity

The haemolytic activity of *L. plantarum K16* strain was tested as previously described Angmo et al., (2016). Briefly, Columbia blood agar plates (Scharlab, Barcelona, Spain) enriched with 5 % of sheep blood were used to grow the microorganism for 48 h at 37 °C. The haemolytic activity was considered positive when a halo was observed in the plates.

2.2.5. Antimicrobial activity

The antimicrobial effect of *L. plantarum K16* was tested against common pathogens such as *Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes* using the agar disk-diffusion method (Abedi, Feizizadeh, Akbari, & Jafarian-Dehkordi, 2013). The pathogenic microorganisms were grown overnight in Brain-Heart Infusion media (Sigma-Aldrich) and spread in Mueller Hinton agar (Sigma-Aldrich). *L. plantarum K16* strain was grown overnight in MRS broth and centrifuge at 12000 rpm for 15 min to evaluate the antimicrobial effect of the biomass and the supernatant. A 6 mm diameter filter paper disc (Scharlab, Barcelona, Spain) was covered separately with 20 μl of cell-free supernatant and the microbial biomass was resuspended in sterilised water. Additionally, the antimicrobial effect of *L. plantarum K16* was also assessed using the agar well diffusion method as previously described by Balouiri, Sadiki, and Ibnsouda (2016). The pathogenic bacteria were spread in Mueller Hinton agar following the same steps as in the agar disk-diffusion method. In this case, a hole of 6 mm was performed and 50 μl of a solution of *L. plantarum K16* strain biomass resuspended in sterilised water were added.

2.3. Experimental design for the study of the factors affecting GABA production

An OFAT experimental design was used to study GABA production by *L. plantarum K16* strain. The GABA production optimisation process was carried out systematically by changing different levels of one factor at fixed levels of the other factors. Incubation temperature, yeast extract concentration as nitrogen source, and fermentation time were selected as main factors affecting GABA production.

As explained below, UHPLC-MS was used to determine the amount (mg/L; ± 0.01) of GABA produced by *L. plantarum K16* in the fermented media under different conditions. In addition, the pH value reached by the fermented medium was measured (±0.1) with a Crison Basic 20 pHmeter (Crison, Barcelona, Spain) and the microbial growth was determined by plating serial dilutions in MRS agar and counting colonies to calculate the colony forming units (CFU) and express as log CFU/mL (±0.01).

2.3.1. Incubation temperature

According to previous studies (Gharehyakheh, 2021; Kwon & Lee, 2018; Tung, Lee, Liu, & Pan, 2011), three incubation temperatures were tested: 30 °C, 34 °C and 36 °C. MRS broth with 17 g/L of yeast extract, enriched with 5 g/L of glucose and 2 mL/L of Tween 80 was used for fermentation assay. In addition, the pH was adjusted to 5.5 and the culture medium was sterilised in autoclave at 121 °C for 20 min. Subsequently, monosodium glutamate (MSG) was supplied to the sterilised medium to obtain a concentration of 500 mM, and, after that, the medium was inoculated with 1 % of *L. plantarum K16* strain. According to previous studies performed by Zarei, Nateghi, Eshaghi, and Abadi (2020), Zhang, Zeng, Tan, Tang, and Xiang (2017) and Di Cagno et al. (2010), *L. plantarum* strains produce the highest amount of GABA after 72 h of incubation. Therefore, samples were taken at this time and the pH, microbial growth and the amount of GABA were measured.

2.3.2. Yeast extract concentration

Yeast extract was chosen as nitrogen source for the fermentation process (Kim, Kim, & Ra, 2021; Kittibunchakul, Yuthaworawit, Whanmek, Suttisansanee, & Santivarangkna, 2021; Wang et al., 2018) and 4, 7, 12 and 17 g/L of yeast extract concentrations were studied. In this case, the culture medium was composed of MRS broth enriched with 5 g/L of glucose, 2 mL/L of Tween 80 and 500 mM of MSG, the initial pH was adjusted to 5.5 and the medium was inoculated with 1 % of *L. plantarum K16*. According to the results derived from the incubation temperature assays, the fermentation was carried out at 34 °C and, as before, samples of the fermented medium were taken after 72 h.

2.3.3. Fermentation time

In addition to the fermentation time used in the incubation temperature and yeast extract concentration assays (72 h), three new fermentation times were tested: 24, 48 and 96 h. The culture medium was prepared from MRS broth 5 g/L of glucose, 2 mL/L of Tween 80, 500 mM of MSG, the initial pH was adjusted to 5.5, and the medium was inoculated with 1 % of *L. plantarum K16* strain. In according to the results derived from the yeast extract concentration and incubation temperature assays, 12 g/L of yeast extract were added to the medium and 34 °C was used for incubation.

2.4. Analysis of GABA by UHPLC-MS

An ACQUITY UPLC H-class system (Waters, Milford, MA, USA) with a HILIC column (130 Å pore size; 1.7 μm particle size; 2.1 mm internal diameter; 100 mm length) (Waters) coupled with a SecurityGuard ULTRA Cartridge pre-column (Waters) was used for the analysis of GABA in the different fermented medium samples. Column temperature was set to 30 °C, sample temperature was set to 10 °C, and injection volume was 3 μl. An isocratic elution with a mixed in volume of 5 % of acetonitrile (HPLC grade, Scharlab, Barcelona, Spain) and 95 % of 0.1 % formic acid (LC-MS grade, Scharlab) prepared in Milli-Q water as mobile phase, and a flow rate of 0.25 mL/min, was used. A triple quadrupole MS equipped with an orthogonal electrospray ionisation source (ESI) ACQUITY TQD (Waters) was used for GABA detection. The instrument operated in electrospray in positive mode (ESI +), and the following MS settings were used: capillary voltage 3.05 kV, desolvation temperature 400 °C, source temperature 120 °C, cone and desolvation gas (nitrogen) flow 60 L/h and 800 L/h, respectively, and collision gas (argon) flow 0.10 mL/min. High purity nitrogen and argon were used (Nippon Gases, Madrid, Spain). MS was run in multiple reaction monitoring (MRM) including two ion transitions for GABA: m/z 104 > 87 for quantification and m/z 104 > 69 for identification. Data acquisition and quantification were performed using MassLynx software version 4.1 (Waters). Quantification was performed against a linear (1/x weighted) regression curve based on the duplicate injection of calibration GABA standard solutions.

2.5. Statistical analysis

IBM-SPSS statistics software version 25.0 (IBM, New York USA) was used for statistical analysis. One-way analysis of variance (ANOVA) was applied to determine the presence of statistically significant differences in the amount of GABA and microbial growth among the fermented samples from different incubation temperatures, yeast extract concentrations, and fermentation times, respectively. Bonferroni's method was used for pairwise comparison. In addition, Pearson correlation coefficient was calculated to investigate the relationship between the amount of biomass obtained after the fermentation treatments and the amount of GABA produced in the fermented samples. Statistical significance was declared at $P \leq 0.05$.

3. Results and discussion

3.1. Safety and probiotic ability of L. plantarum K16 strain

3.1.1. Carbohydrates metabolism

As it has been previously reported, different types of carbohydrates are processed in the large intestine producing beneficial health effects such as increase minerals absorption, modulate glucose, or decrease cholesterol levels (Seal, Courtin, Venema, & de Vries, 2021). Carbohydrates also can play a key role in the gut microbiota preservation and, thus, in the prevention of gastrointestinal or cardiovascular diseases (Hugenholtz, Mullaney, Kleerebezem, Smidt, & Rosendale, 2013). Furthermore, carbohydrates metabolism by LAB could lead to produce several postbiotic compounds such as organic acids, exopolysaccharides, or short-chain fatty acids (Wang et al., 2021).

The ability of *L. plantarum K16* to process 49 types of carbohydrates was assessed using API 50 CHL strips. Table 1 shows that this strain can metabolise monosaccharides, like glucose, galactose, fructose, mannose, arabinose and ribose, and monosaccharides derived compounds such as N-acetylglucosamine. All these compounds are easily use as a source of energy to enhance gut microbial growth (Hedberg, Hasslof, Sjostrom, Twetman, & Stecsen-Blicks, 2008). In addition, *L. plantarum K16* strain can degrade disaccharides such as cellobiose, melibiose, trehalose, gentibiose and turanose, as well as glucosides like amygdaline, arbutin, esculin and salicin (Table 1). Gebreselassie, Abay, and Beyene (2016) reported that a *L. plantarum strain* isolated from naturally fermented buttermilk could catabolise all these carbohydrates, except amygdaline. Contrarily, Menon, Munjal, and Sturino (2015) highlighted the ability of a *L. plantarum* strain to catabolise amygdaline using it as a carbon and energy source. The use of amygdaline by this strain could be considered an essential probiotic ability because this sugar is classified as a cytotoxic cyanogenic glycoside that could enhance the degeneration of nerves. Furthermore, *L. plantarum K16* could also degrade sweeteners, like mannitol and sorbitol, oligosacharides like melezitose and raffinose, and the polysaccharide inulin (Table 1). The catabolism of these carbohydrates could have different beneficial human health effects. For instance, Xiao, Metzler-Zebeli, and Zebeli (2015) indicated that the degradation of mannitol and sorbitol could enhance the digestion process, increase the absorption of nutrients, stimulate the synthesis of lactic and butyric acid, and persevere a healthy intestine. Other authors indicated that the inulin degradation in the gut enhances the synthesis of butyric acid, increases the absorption of minerals, protects against gastrointestinal disorders, or stimulates the immune system (Niba, Beal, Kudi, & Brooks, 2009; Shoaib, Shehzad, Omar, Rakha, Raza, Sharif, Shakeel, Ansari, & Niazi, 2016). Likewise, raffinose catabolism could also stimulate the growth of probiotics, lead to increase iron absorption and maintain gut functionality (Mao et al., 2018).

Table 1
Carbohydrates fermentation profiling for *L. plantarum K16* strain obtained by using the Analytical Profile Index (API) based on 49 different fermentable carbohydrates.

Group and Species	*Reaction*	*Group and Species*	*Reaction*
Monosaccharides		**Trisaccharides**	
D-Arabinose	–	D-Melezitose	+
L-Arabinose	+	D-Raffinose	+
D-Ribose	+	**Polysaccharides**	
D-Xylose	–	Inulin	+
L-Xylose	–	Starch	–
D-Lyxose	–	Glycogen	–
D-Tagatose	–	**Glycosyl Compounds**	
D-Fucose	–	Esculin	+
L-Fucose	–	Salicin	+
Methyl-β-D-xylopyranoside	–	Arbutin	+
D-Galactose	+	Amygdaline	+
D-Glucose	+	N-Acetylglucosamine	+
D-Fructose	+	**Polyols**	
D-Mannose	+	Glycerol	–
L-Sorbose	–	Erythritol	–
L-Rhamnose	–	D-Adonitol	–
Methyl-α-D-mannopyranoside	–	Dulcitol	–
Methyl-α-D-glucopiranoside	–	Inositol	–
Disaccharides		D-Mannitol	+
D-Cellobiose	+	D-Sorbitol	+
D-Maltose	+	Xylitol	–
D-Lactose	+	D-Arabitol	–
D-Melibiose	+	L-Arabitol	–
D-Trehalose	+	**Potassium salts of gluconic acid**	
D-Sucrose	+	Potassium gluconate -	
Gentiobiose	+	Potassium 2-ketogluconate	–
D-Turanose	+	Potassium 5-ketogluconate	–

+, positive reaction; –, no reaction.

3.1.2. Enzymatic profiling

Probiotic microorganisms could play a key role in the digestion of several kind of nutrients, including the metabolism of carbohydrates, proteins, or lipids (Stoyanovski et al., 2013; Yi, Pan, Long, Tan, & Zhao, 2020). According to Plaza-Diaz, Ruiz-Ojeda, Gil-Campos, and Gil (2019), *Lactobacillus* species could present more than twenty essential enzymatic activities that could have a strong biological effect in the gastrointestinal tract of humans.

The results of the enzymatic profiling of *L. plantarum K16* strain showed that this microorganism did not present enzymatic activity such as alkaline phosphatase, alkaline esterase, trypsin, α-chymotrypsin,

Table 2
Enzymatic profiling for *L. plantarum K16* strain obtained by using the Analytical Profile Index (API) based on 19 different enzyme activities.

Enzyme	*Substrate*	*Reaction*	*Ammount of hydrolysed substrate (nmoles)*
Alkaline phosphatase	2-Naphthyl phosphate	–	
Alkaline esterase (C8)	2-Naphthyl caprylate	–	
Trypsin	N-Benzoyl-DL-arginine-2-naphthyl amide	–	
α-Chymotrypsin	N-Glutaryl-phenylalanine-2-naphthylamide	–	
α-Galactosidase	6-Br-2-Naphthyl-α-D-Galactopyranoside	–	
β-Glucuronidase	Naphthol-AS-BI- β-D-glucuronide	–	
α-Mannosidase	6-Br-2-Naphthyl-α-D-mannopiranoside	–	
α-Fucosidase	2-Naphthyl-α-L-fucopiranoside	–	
Esterase (C4)	2-Naphthyl butyrate	+	5
Lipase (C14)	2-Naphthyl myristate	+	5
Valine arylamidase	L-Valyl-2-naphthyl amide	+	10–20
Cystine arylamidase	L-Cystil-2-naphthyl amide	+	10–20
Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	+	20–30
Leucine arylamidase	L-Leucyl-2-naphthyl amine	+	>40
Acidic phosphatase	2-Naphthyl-phosphate	+	>40
β-Galactosidase	2-Naphthyl-α-D-Glucopyranoside-β-D-galactopyranoside	+	>40
α-Glucosidase	2-Naphthyl- α-D-glucopyranoside	+	>40
β-Glucosidase	6-Br-2-Naphthyl-β-D-glucopyranoside	+	>40
N-Acetyl-β-glucosaminidase	1-Naphthyl-N-acetyl-β-D-glucosaminide	+	>40

+, positive reaction; -, no reaction.

α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase (Table 2). In this regard, other authors highlighted the relevance of probiotics not presenting β-glucuronidase activity due to this enzyme can degrade glucoronidated compounds into cytotoxic metabolites which can enhance colon carcinogenesis (Arias et al., 2013; Song, Jang, Kim, & Paik, 2019). On the other hand, the results obtained for *L. plantarum K16* showed a slight activity of esterase and lipase (Table 2). Zhang, Liang, He, Feng, and Li (2022b) reported that lipase activity of probiotics in the gut have beneficial effects by increasing the absorption of nutrients, improving metabolism, and maintaining gut structure. Furthermore, *L. plantarum K16* strain showed a high activity for valine arylamidase or cystine arylamidase enzymes with the ability to hydrolase 10 to 20 nmoles of substrate. The enzymatic activity of naphthol-AS-BI-phosphohydrolase of this strain was more intense, showing a hydrolytic activity between 20 and 30 nmoles of substrate. Moreover, the activity of leucine arylamidase and acidic phosphatase was even greater, hydrolysing >40 nmoles of substrate (Table 2). Previous results also reported that a *Lactobacillus* strain isolated from Cheddar cheese showed activity of valine arylamidase, cystine arylamidase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase (Oberg et al., 2016). Jawan et al. (2021) highlighted the importance of leucine arylamidase activity as it is mainly involved in human metabolism degrading leucine into acetyl CoA and acetyl acetate, and that of acidic phosphatase and naphthol-AS-BI-phosphohydrolase activities because they are essential during the digestive process to release phosphorylated groups.

L. plantarum K16 strain also showed high activity (40 nmoles of substrate) for enzymes such as β-galactosidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase (Table 2). These results agree with those reported by Park and Lim (2015) for *L. plantarum FH185* strain isolated from the faeces of healthy adults. In this sense, N-acetyl-β-glucosaminidase could have an antifungal effect because this enzyme could break down chitin found in the cell wall of pathogens such as *Aspergillus niger* (Hassan & Ismail, 2021). Colombo, Castilho, Todorov, and Nero (2018) reported that LAB with high activity of β-galactosidase could be useful to enhance the degradation of lactose and, thus, reduce its intolerance of lactose.

3.1.3. Antibiotic susceptibility

LABs have been primarily classified as GRAS microorganisms but nowadays it is critical to evaluate safety issues such as antibiotic resistance. Therefore, it is important to determine the susceptibility of probiotics to antibiotic therapy and to assess whether their resistance to antibiotics could be horizontally transmitted (Erginkaya, Turhan, & Tatlı, 2018). Table 3 shows the susceptibility of *L. plantarum K16* against 12 antibiotics with different mechanisms of action. As observed, this strain presents high sensibility against rifampicin, tetracycline and other antibiotics that inhibit the synthesis of proteins such as erythromycin and chloramphenicol. These results agree with those reported previously indicating that *Lactobacillus* species are generally susceptible to protein synthesis inhibitors such as erythromycin, tetracycline, chloramphenicol, and clindamycin (Gueimonde & Sánchez, 2013). Contrarily, *L. plantarum K16* strain was resistant against clindamycin producing an inhibitor halo of 11.0 mm. Likewise, this strain showed resistance against ofloxacin that inhibits topoisomerase type II and metronidazole and ciprofloxacin that block the synthesis of metabolic factors (Table 3). However, intermediate resistance was observed against trimethoprim, which also can block the synthesis of metabolic factors. Furthermore, sensitivity to ampicillin, classified as an antibiotic inhibitor of cell wall synthesis, was verified with an inhibitor halo of 26.0 mm. On the other hand, *L. plantarum K16* was resistant against other antibiotics that inhibit cell wall synthesis such as penicillin and vancomycin (Table 3). In this regard, Ouwehand, Forssten, Hibberd, Lyra, and Stahl (2016) indicated that *Lactobacillus* species normally present resistance against vancomycin, which is considered as a non-transmissible natural resistance, and clindamycin. Nevertheless, resistance to ampicillin has not commonly been found in LAB. Several studies have highlighted that probiotic with specific antibiotic resistances could be useful to be co-administered with an antibiotic therapy because they can help in the maintenance of the microbiota structure through the stimulation of the immune system, preserving the intestinal barrier or avoiding pathogens colonisation (Machado et al., 2022; Ouwehand et al., 2016; Yu et al., 2013). In this case, to satisfy the guidance of the European Food Safety Authority (EFSA) and to deeply evaluate the antimicrobial resistance, further studies are required to determine the minimum inhibitory concentration of the evaluated antibiotics and assess the molecular characterization of the antimicrobial resistance genes to determine the likelihood to be transmitted (EFSA, 2012; Ayala et al., 2019).

Table 3
Susceptibility of *L. plantarum K16* strain to 12 different antibiotics.

Antibiotic type	*Antibiotic compound*	*Antibiotic amount (μg)*	*Halo diameter (mm)*	*Susceptibility*
Penicillins	Ampicillin	10	26 ± 1.0	Sensitive
Amphenicols	Chloramphenicol	30	23 ± 0.6	Sensitive
Macrolides	Erythromycin	15	22 ± 1.0	Sensitive
Rifampicins	Rifampicin	5	22 ± 2.0	Sensitive
Tetracyclines	Tetracycline	30	21 ± 0.6	Sensitive
Sulfonamides	Trimethoprim	5	18 ± 0.6	Intermediate
Penicillins	Penicillin	2[1]	15 ± 0.6	Resistant
Lincosamides	Clindamycin	2	11 ± 0.6	Resistant
Glycopeptides	Vancomycin	30	nd	Resistant
Quinolones	Ciprofloxacin	5	nd	Resistant
Miscellaneous antibiotics	Metronidazole	5	nd	Resistant
Quinolones	Ofloxacin	5	nd	Resistant

[1] units; nd, not detected.

3.1.4. Haemolytic activity

The haemolytic activity is considered a virulence factor generally produced by haemolysing protein, which triggers the lysis of the red blood cell membrane. The results of haemolytic activity test can be classified as Alpha haemolysis (green halo associated to partial lysis), Beta haemolysis (yellowish halo related to the full lysis), and Gamma haemolysis (lack of lysis) (Savardi, Ferrari, & Signoroni, 2018). In this study, *L. plantarum K16* strain showed Gamma haemolysis, i.e., no haemolysis activity. This result agrees with that reported by Halder, Mandal, Chatterjee, Pal, and Mandal (2017) for *L. plantarum* strains isolated from cow milk curd.

3.1.5. Antimicrobial activity

The antibacterial effect of probiotics has gained interest due to its potential to be used as safe bio-preservatives, which are easily degraded into the gastrointestinal tract (Botthoulath, Upaichit, & Thumarat, 2018). Furthermore, it has been reported that the antimicrobial activity of *Lactobacillus* could be an alternative for antibiotic treatments and, thus, avoid antibiotic resistances (Jimenez-Trigos et al., 2022). In this regard, LAB could have a bactericidal effect producing several postbiotic metabolites such as organic acids, peptides or bacteriocins (Liu, Zhang, Yang, & Huang, 2015; Sharma et al., 2017). Table 4 showed that *L. plantarum K16* strain did not have an inhibitory effect against any of the pathogen bacteria in the cell-free supernatant substrate using the disk-diffusion method. Contrarily, the microbial biomass produced an inhibition halo of 8.3 mm diameter against *E. coli.* The results of the agar well diffusion test showed an inhibition halo of 11 mm diameter when *L. plantarum K16* was in contact with the Gram-negative bacilli, *E. coli* and *S. typhimurium* (Table 4). Amarantini, Budiarso, Antika, and Prakasita (2020) and Divyashree, Anjali, Somashekaraiah, and Sreenivasa (2021) indicated that *L. plantarum* isolated from different fermented foods presented antimicrobial activity against *Salmonella* species, which could be useful to prevent and treat food-borne illnesses. Likewise, other

Table 4
Antimicrobial activity of *L. plantarum K16* strain against three different pathogens determined by disk-diffusion and agar well diffusion methods. Inhibition zone is expressed as halo diameter.

		Halo diameter (mm)		
	Substrate	***E. coli***	***S. typhimurium***	***L. monocytogenes***
Disk-diffusion method	Microbial biomass	8.3 ± 0.6	nd	nd
	Cell-free supernatant	nd	nd	nd
Agar well diffusion method	Microbial biomass	11.0 ± 1.4	11.0 ± 1.4	nd

nd, not detected.

authors reported that *L. plantarum* strains highly inhibited *E. coli* protecting against the development of diarrhea and maintain a healthy gastrointestinal tract (Ali, Shyum Naqvi, & Yousuf, 2020; Pazhoohan, Sadeghi, Moghadami, Soltanmoradi, & Davoodabadi, 2020). In this case, to ensure that *L. plantarum K16* can protect against pathogenic bacteria, more research is needed. For instance, the comparison of the inhibition halos diameters obtained in presence of *L. plantarum K16* and a known inhibitory substance against *E. coli, S. typhimurium* and *L. monocytogenes*. As well as the evaluation of the competitive exclusion in broth culture or the attachment and competition using cell culture techniques (Ayala et al., 2019; Jamyuang et al., 2019).

3.2. GABA production by L. plantarum K16 strain

3.2.1. Incubation temperature

Incubation temperature is a major parameter that mainly affects the growth dynamics of the probiotic microorganisms. For the optimal production of GABA, the adjustment of the incubation temperature is essential to maintain the thermodynamic equilibrium of the GAD biosynthetic pathway (Dhakal, Bajpai, & Baek, 2012). In the present work, the GABA production by *L. plantarum K16* incubated at 30 °C was 421.96 ± 43.12 mg/L, and the amount of microbial growth was significantly ($P \leq 0.05$) higher compared to that produced at 34 °C or 36 °C (Table 5). When incubation temperature increased from 30 °C to 34 °C, the bioconversion of MSG to GABA was enhanced, reaching the amount of 561.36 ± 28.26 mg/L of GABA, a pH value of the fermented media of 4.4 ± 0.07, and a significantly lower ($P \leq 0.05$) microbial growth. Likewise, the highest incubation temperature of 36 °C significantly ($P \leq 0.05$) reduced the biocatalytic activity and thus, the amount of GABA produced was lower, 329.25 ± 9.31 mg/L, as well as the microbial growth decreased (Table 5). Furthermore, no significant correlation ($P > 0.05$) was observed between the biomass production and the amount of GABA obtained in the range of incubation temperatures used.

According to the above-mentioned results, 34 °C could be considered the optimal incubation temperature for producing the highest amount of GABA by *L. plantarum K16* strain, which agrees with other previous studies (Tung et al., 2011) that obtained the highest GABA yield (around 770 mg/L), at 34 °C by a *L. plantarum* strain. Contrarily, other authors used different *L. plantarum* strains and reported different optimal incubation temperatures for GABA production. For instance, Tajabadi et al. (2015) performed an optimisation process of GABA production using an *L. plantarum* Taj-Apis362 strain isolated from honeybees that obtained at 37 °C the highest amount of GABA (250 mg/L). On the other hand, Zhang et al. (2017) isolated an *L. plantarum BC114* strain from Chinese paocai and determined that 30 °C was the best temperature to increase the GABA yield using a single factor optimisation process.

3.2.2. Yeast extract concentration

Yeast extract is one of the most suitable nitrogen sources for LAB growth due to its high protein concentration and, thus, the high availability of essential amino acids (Jacob, Hutzler, & Methner, 2019). Yeast extract also presents a high concentration of vitamin B complex and a wide variety of nucleic acids such as guanosine 5′-monophosphate or inosine 5′-monophosphate (Song, Lee, Lee, & Baik, 2021). In addition, previous studies have reported that the yeast extract can enhance more the production of GABA than other nitrogen sources (Chen, Xu, & Zheng, 2015; Park, Kim, Kang, Shin, Yang, Yang, & Jung, 2021b).

Table 5 shows the production of GABA, pH, and the microbial growth at different yeast extract concentration. As observed, *L. plantarum K16* strain produced 172.35 ± 10.25 mg/L of GABA and a microbial growth near to 9 log CFU/mL when 4 g/L of yeast extract were used in the culture medium. However, the production of GABA raised ($P \leq 0.05$) up to 359.61 ± 45.39 mg/L whereas the microbial growth significantly decreased to 8.54 ± 0.09 log CFU/mL when yeast extract concentration was7 g/L. Highest GABA production was reached when yeast extract

Table 5
Effect of the incubation temperature, yeast extract concentration and incubation time on the amount (mean ± standard deviation) of GABA (mg/L), viable counts (log CFU/mL) and pH by *L. plantarum K16* strain in MRS broth.

Optimization of the incubation temperature					
Incubation temperature (℃)	***Yeast extract concentration (g/L)***	***Incubation time (h)***	***GABA (mg/L)***	***Viable counts (Log CFU/mL)***	***pH***
30	17	72	421.96 ± 43.12^{b}	9.11 ± 0.11^{a}	4.31 ± 0.02
34	17	72	561.36 ± 28.26^{a}	7.44 ± 0.06^{b}	4.40 ± 0.07
36	17	72	329.25 ± 9.31^{c}	6.79 ± 0.16^{c}	4.22 ± 0.01
Optimization of the yeast extract concentration					
Incubation temperature (℃)	***Yeast extract concentration (g/L)***	***Incubation time (h)***	***GABA (mg/L)***	***Viable counts***	***Incubation temperature (℃)***
34	4	72	172.35 ± 10.25^{d}	8.96 ± 0.07^{a}	4.51 ± 0.01
34	7	72	359.61 ± 45.39^{c}	8.54 ± 0.09^{b}	4.40 ± 0.02
34	12	72	816.84 ± 22.44^{a}	7.94 ± 0.06^{c}	4.42 ± 0.01
34	17	72	561.36 ± 25.26^{b}	7.44 ± 0.06^{d}	4.40 ± 0.07
Optimization of the incubation time					
Incubation temperature (℃)	***Yeast extract concentration (g/L)***	***Incubation time (h)***	***GABA (mg/L)***	***Viable counts***	***Incubation temperature (℃)***
34	12	0	15.95 ± 0.80^{d}	7.44 ± 0.08^{d}	5.50 ± 0.01^{a}
34	12	24	189.29 ± 33.82^{c}	9.47 ± 0.03^{a}	4.36 ± 0.01^{b}
34	12	48	274.16 ± 44.16^{c}	8.58 ± 0.09^{b}	4.36 ± 0.01^{b}
34	12	72	816.84 ± 22.44^{b}	7.94 ± 0.06^{c}	4.42 ± 0.01^{b}
34	12	96	1000.23 ± 70.82^{a}	6.99 ± 0.03^{e}	4.42 ± 0.01^{b}

[a, b, c,d]Means with different superscripts indicate statistically significant ($P \leq 0.05$) differences in the same column for the different parameters studied.

concentration was 12 g/L (816.84 ± 22.44 mg/L), a pH media of 4.4 ± 0.01, and a microbial cell growth concentration of 7.94 ± 0.06 log CFU/mL. However, a higher concentration of yeast extract (17 g/L) reduced significantly ($P \leq 0.05$) the GABA production by *L. plantarum K16* strain (Table 5). Similarly, Binh, Ju, Jung, and Park (2014) reported that an increase in yeast extract supplementation to MRS broth from 20 to 40 g/L resulted in a decrease of GABA production by *L. brevis*. Likewise, Wang et al. (2018b) reported that a yeast extract concentration higher than 25 g/L resulted in lower GABA production by *L. brevis NCL912* strain. In the present study, the GABA synthesis by *L. plantarum K16* was significantly ($P \leq 0.05$) inverse correlated to the microbial cell growth (-0.721). Therefore, a high production of GABA is strongly correlated with a low microbial growth. This correlation suggests that a higher concentration of yeast extract stimulates the GAD pathway of *L. plantarum K16* focusing the metabolism on the production of higher amount of GABA but not in duplication.

3.2.3. Fermentation time

As it is well known, microbial cell growth is generally divided into four well-differentiated phases: lag phase, exponential growth, stationary phase, and exponential decay. Growth kinetics of *L. plantarum* strains is characterised due to the production of organic acids, mainly lactic acid, triggered by the consumption of carbohydrates during the exponential growth. The high concentration of lactic acid decreases the media pH and leads to a stationary phase (Charalampopoulos, Pandiella, & Webb, 2002; Rezvani, Ardestani, & Najafpour, 2017). The depletion of nutrients and the high concentration of toxic metabolic products in the stationary phase generates a stressful environment and, thus, the microorganism death rate increases. Meanwhile, LABs have developed several protective mechanisms against stressful situations by activating several regulons when the microorganisms go from the exponential to the stationary phase. For instance, the GAD pathway is considered an important mechanism triggered against osmotic, acid or starvation stress (Papadimitriou et al., 2016). In this sense, several studies have reported that GABA production by *L. plantarum* strains could increase at the end of the exponential phase or near the stationary phase (Park et al. 2021b). Likewise, Rayavarapu, Tallapragada, and Ms (2021) observed that during the first 24 h of incubation, LABs focused on cell multiplication, and the GABA yield was low but after 48 h the microorganisms reached the stationary phase and the amount of GABA produced was higher.

The time associated with each growth phase is close related to the strain used for the experiment and, in the present study, the fermentation time was extended to 96 h. The results showed that after 24 h of incubation, the microbial growth significantly ($P \leq 0.05$) increased coupled with a dramatic decrease of the pH media (4.36 ± 0.01), and the GABA produced (189.29 ± 33.82) was not significant in comparison with the initial conditions (Table 5). From 24 to 48 h, the amount of GABA slightly increased ($P > 0.05$) to 274.16 ± 44.16 mg/L coupled with a significant ($P \leq 0.05$) decrease of the microbial cell growth. A significant ($P \leq 0.05$) decrease in the *L. plantarum K16* growth was shown as fermentation time increased, together with a significant ($P \leq 0.05$) increase of the amount of GABA. The highest amount of GABA produced by *L. plantarum K16* strain was achieved after 96 h (1000.23 ± 70.82 mg/L) (Table 5). Similar results were reported by other authors using different *L. plantarum* strains (Sharma et al., 2021; Fuming, Chen Jian, & Xiaoran, 2017). In addition, a significant ($P \leq 0.05$) strong inverse correlation between GABA and microbial growth (−0.933) was obtained. Therefore, an increase of the amount of GABA significantly decreases the microbial cell growth during fermentation. This relationship could be due to the decrease of nutrients coupled with the increase of organic acids, which increased the microbial stress reducing the cell viability but, this stressful environment, could enhance the activation of the GAD pathways and thus, increases the GABA synthesis (Rayavarapu et al., 2021).

4. Conclusions

L. plantarum K16 strain isolated from Kimchi has demonstrated probiotic ability with potential to enhance the digestion and absorption of different kind of nutrients, stimulate the synthesis of beneficial compounds and it could have an inhibitory effect against pathogenic bacteria. Furthermore, these results should encourage to perform further characterisation studies to deeper assess the safety and probiotic effect of *L. plantarum K16* strains. Focusing on the production of GABA, *L. plantarum K16* showed that it is strongly influenced by the incubation temperature, the concentration of yeast extract and the fermentation time. In this regard, MRS broth enriched with 5 g/L of glucose, containing 12 g/L of yeast extract and 500 mM of MSG, adjusted to an initial pH of 5.5, inoculated with 1 % of *L. plantarum K16* strain and incubated at 34 °C for 96 h produced up to 1000 mg/L of GABA. Further optimisation of GABA production should be performed assessing other parameters involved in the GAD biosynthetic pathway. Despite more research being needed, the results suggest that L. plantarum K16 and the amount of GABA produced could potentially be used as functional ingredients.

CRediT authorship contribution statement

Lucía Diez-Gutiérrez: Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing. **Leire San Vicente:** Investigation, Resources, Writing – review & editing. **Jessica Sáenz:** Investigation, Writing – review & editing. **Luis Javier R. Barron:** Formal analysis, Writing – review & editing. **María Chávarri:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics statement

The authors declare no ethical issue related with this article.

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ANNEX I.III: PUBLICATION

scientific reports

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Biosynthesis of gamma-aminobutyric acid by *Lactiplantibacillus plantarum K16* as an alternative to revalue agri-food by-products

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Probiotic metabolites, known as postbiotics, have received attention due to their wide variety of promoting health effects. One of the most exciting postbiotic is gamma-aminobutyric acid (GABA), widely produced by lactic acid bacteria, due to its benefits in health. In addition, the performance of the biosynthesis of GABA by *Lactiplantibacillus plantarum* could be modulated through the modification of fermentation parameters. Due to their high nutritional value, agri-food by-products could be considered a useful fermentation source for microorganisms. Therefore, these by-products were proposed as fermentation substrates to produce GABA in this study. Previously, several experiments in Man Rogosa Sharpe (MRS) broth were performed to identify the most critical parameters to produce GABA using the strain *Lactiplantibacillus plantarum K16*. The percentage of inoculum, the initial pH, and the concentration of nutrients, such as monosodium glutamate or glucose, significantly affected the biosynthetic pathway of GABA. The highest GABA yield was obtained with 500 mM of monosodium glutamate and 25 g/L of glucose, and an initial pH of 5.5 and 1.2% inoculum. Furthermore, these investigated parameters were used to evaluate the possibility of using tomato, green pepper, apple, or orange by-products to get GABA-enriched fermented media, which is an excellent way to revalorise them.

Probiotic microorganisms are now widely consumed worldwide due to their potential to preserve and enhance human health[1] through their direct effect on the intestinal microbiota, modulation of the immune system, protection against pathogens colonisation, or reduction of oxidative stress, among others[2]. These health benefits can be produced because of the positive interaction between probiotics and the host gut microbiota, triggering the activation of different intracellular signalling pathways[3]. For example, the activation of genes involved in the synthesis of mucin avoids pathogens' adhesion to the gut barrier, the enhancement of phagocytosis through the increase of macrophages or the attenuation of pro-inflammatory cytokines production[4].

Likewise, probiotics can also produce host benefits by metabolising different nutrients and producing bioactive compounds classified as postbiotics which can be defined as metabolites synthesised by these microorganisms or other compounds released during fermentation processes[5–9]. A wide range of compounds could be classified as postbiotics, such as vitamins, minerals, amino acids, neurotransmitters, or lipid compounds[10]. One of the most promising postbiotic is the neurotransmitter gamma-aminobutyric acid (GABA)[11,12]. This compound can reduce anxiety and depression in humans, influence several neurochemical pathways, enhance the immune system, or modulate blood pressure decreasing the likelihood of developing heart problems[13].

Consistent with the health benefits of GABA, this compound was initially produced industrially by chemical synthesis to meet pharmaceutical and food companies' demands[14]. However, the poor synthesis performance, the detrimental effect on the environment, and the low profitability of the process led to the substitution of the chemical production with a more suitable production by using a biotechnological process carried out by

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microorganisms[15]. Some examples of interesting GABA producers are lactic acid bacteria (LAB)[16], *Bacillus subtilis*[17], *Aspergillus oryzae*[18], *Listeria monocytogenes*[19] *or Bifidobacterium*[20]. Among these microorganisms, LABs have been considered one of the most attractive alternatives to synthesise GABA due to the high performance of their biosynthetic process, their classification as generally regarded as safe (GRAS) microorganisms and their potential beneficial effects on human health[21].

The synthesis of GABA is commonly performed through the glutamic acid decarboxylase pathway (GAD) as a mechanism triggered under stressful environments. Specifically, a molecule of L-glutamic acid (L-Glu) is decarboxylase by a GAD enzyme resulting in the production of a GABA molecule[22]. Usually, the GAD enzyme is encoded by a *gadB* gene, but some LAB can present two genes such as *Levilactobacillus brevis* which has a *gadA* and *gadB* gene. Moreover, some species, such as *Lactobacillus buchneri, Lb. curvatus or Lb. sakei,* could even present potential transcriptional regulators that can enhance the synthesis of GABA[21]. For instance, Gong et al.[23] highlighted how the transcriptional regulator GadR presented in *L. brevis* is directly linked to the high GABA yield and the resistance against acid environments of this bacteria. Due to the diversity of genetics involved in the GAD system, the GABA yield could be very different between species such as, *L. buchneri WPZ001* yielded 117 g/L of GABA[24], *L. brevis NCL912* produced 103.7 g/L of GABA[25] or *Lactiplantibacillus plantarum (Lactobacillus plantarum) N5* yielded 21.8 g/L of GABA[26]. Cui et al.[21] explained that the GAD systems is strain-specific and even strains with the same GAD system could have different yield of GABA.

Within LAB, *L. plantarum* strains could produce a great amount of GABA, depending on the source where they were isolated from, and the yield of the machinery involved in the biosynthetic pathway of GABA[27].

GAD pathway performance can be modulated by adjusting several environmental parameters such as temperature, initial pH, or oxygen availability. In addition, the type and concentration of minerals, coenzymes, nitrogen, carbon sources, and other additives could positively influence GABA biosynthesis[28]. Several studies have been conducted to adjust the main physic-chemical parameters involved in the GABA synthesis. For instance, Sharafi and Nateghi[29] optimised the GABA production by *L. brevis* by studying the effect of temperature, initial pH, L-Glu, concentration and fermentation time. They obtained that the fermentation carried out at 34 °C, with an initial pH of 4.65, 650 mmol of L-Glu and for 96 h of incubation time, enhanced more than twice the synthesis of GABA compared to non-optimised conditions. Wu et al.[30] and Song and Yu[31] reported that the inoculum percentage and the nitrogen and carbon source type could positively influence the GABA synthesis by *Lactobacillus* strains.

Furthermore, these optimisation processes are generally performed using MRS broth, characterised by the high concentration of nutrients necessary for *Lactobacillus* growth. However, the wide variety of nutrients used in this culture media increases the cost of the production process. Thus, it is not considered a suitable fermentation media for scale-up production[32]. During the last years, by-products from the agri-food industry has gained attention to be used as low-cost fermentation media which puts a value on potential pollutants.

In general, agri-food industries generate a considerable amount of waste mainly produced from the transformation of raw fruits and vegetables into final products like juices or smoothies, which normally discard structural parts such as seeds, peels, leaves, or pulps. Mnisi et al.[33] reported that from 900 million metric tons of fruit production in 2020, approximately a 30% was discarded, normally producing a strong environmental impact because these by-products are normally burned or placed in landfills[34], although it is also being used to produce animal feed[35]. Consequently, the use of these agri-food by-products as culture media for fermentation processes can be a good way to revalorise this type of waste, as well as to produce bioactive compounds useful for the formulation of new drugs and functional foods[36].

Falah et al.[37] proposed to use molasses, dairy sludge, and soybean meal as fermentation media to produce GABA by *L. brevis, Limosilactobacillus fermentum* and *L. plantarum*. Zarei et al.[38] made a functional drink using whey protein, considered a high environmental impact waste product, as the primary source to synthesise GABA by *L. plantarum*. In our previous study, *L. plantarum K16* was isolated from Kimchi and identified as GABA-producer. Then, it was evaluated how parameters such as temperature, the concentration of yeast extract and incubation time influenced the GABA production by *L. plantarum K16*[39] Therefore, the objective of this study was to continue with the analysis of parameters, such as inoculum percentage, initial pH, monosodium glutamate (MSG) concentration, and glucose concentrations, involved in the GABA production of *L. plantarum K16* using MRS broth and achieve the highest yield of GABA in this medium. Afterwards, a fermentation trial was performed to determine if tomato, green pepper, apple, or orange by-products could be considered as suitable fermentation substrates to obtain GABA-rich fermented products.

Methods

Microbial strain. LABs were isolated from kimchi through standard culturing methods in the Food Biotechnology laboratory (TECNALIA, Miñano, Spain). The isolated LABs were grown in MRS broth supplied with L-Glu, and the supernatants were collected to analyse the GABA content using ultra-high-performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS). Only one of the isolated LABs was able to produce GABA which was identified as *L. plantarum K16*. Therefore, *L. plantarum K16* was used to evaluate how different parameters could modulate the synthesis of GABA.

GABA production by *L. plantarum* K16 strain. The optimisation process for GABA synthesis by *L. plantarum K16* strain was carried out in several stages following a one-factor-at-a-time (OFAT) experimental design in MRS broth (Sigma-Aldrich, Madrid, Spain). Therefore, the optimisation process was performed by studying different levels of one fermentation parameter while keeping unchanged the other fermentation parameters. The beginning of this optimisation process was explained in a previous study[39], where the incubation temperature, concentration of yeast extract and fermentation time were evaluated. The results of this experiments

indicated that the initial conditions to continue the optimisation process should be MRS broth supplied with 5 g/L of glucose, 12 g/L of yeast extract, an initial pH of 5.5, inoculum of 1%, 500 mM of MSG, incubation temperature of 34 °C and 96 h of fermentation. Furthermore, in the current research the fermentation parameters studied in MRS broth were inoculum percentage, initial pH, MSG concentration and glucose concentrations. For each experiment, an inoculum of *L. plantarum K16* was prepared in MRS broth overnight at 37 °C. Then, the amount of GABA production (mg/L; ± 0.01) was quantified by UHPLC-MS. Likewise, the microbial growth was measured by plating serial dilutions in MRS agar and counting colonies to calculate the colony-forming units (CFU) and expressed as log CFU/mL (± 0.01). Finally, the pH value of the fermented medium was measured (± 0.1) with a Crison Basic 20 pHmeter (Crison, Barcelona, Spain).

Inoculum percentage. According to the research of Kantachote et al.[40], different percentages of *L. plantarum K16* strain were used in the fermentation process. The fermentation media was prepared, with 250 mL Erlenmeyer flask containing 100 mL of working volume, by adding 5 g/L of glucose to MRS broth composed by 12 g/L of yeast extract, adjusted to an initial pH of 5.5 and sterilised by autoclaving the culture medium at 121 °C for 15 min. Afterwards, the medium was enriched with 500 mM of sterilised MSG, further inoculated with 0.8, 1.0, 1.2 and 1.4% of *L. plantarum K16* strain and incubated at 34 °C without shaking. After 96 h of fermentation, analytical samples of the fermented medium were taken to determine the pH, GABA amount and the CFU/mL.

Initial pH. The MRS broth was prepared as previously described for glucose, yeast extract and MSG concentrations, and 4.0, 4.5, 5.5 and 6.0 as different initial pH values. An inoculum percentage of 1.2% was selected as the optimum value obtained for GABA production from the previous stage, and the fermentation medium was incubated at 34 °C during 96 h. Likewise, analytical samples of the fermented medium were taken to determine the pH, GABA amount and the CFU/mL.

MSG concentration. Different MSG concentrations (100, 300, 500 and 550 mM) were evaluated to determine how this precursor of the GAD biosynthetic pathway could influence the production of GABA. The fermentation media was prepared by adding 5 g/L of glucose to MRS broth composed by 12 g/L of yeast extract. Following the results obtained in the previous OFAT stages, the initial pH was fixed to 5.5, the medium was inoculated with 1.2% of *L. plantarum K16* strain, and the medium was incubated at 34 °C during 96 h. Also, analytical samples of the fermented medium were taken to determine the pH, GABA amount and the CFU/mL.

Glucose concentration. According to the scientific literature, glucose was chosen as the best carbon source for the optimisation of GABA production by LAB fermentation[30,41]. Different glucose concentrations (20, 23, 25 and 27 g/L) were tested in the MRS broth containing 12 g/L of yeast extract and 500 mM of MSG. This MSG concentration was selected as the optimum value obtained for GABA production from the previous section. As other assays, initial pH was adjusted to 5.5, the medium was inoculated with 1.2% of *L. plantarum K16* strain and incubated at 34 °C for 96 h. Moreover, analytical samples of the fermented medium were taken to determine the pH, GABA amount and the CFU/mL.

GABA production using agri-food by-products. The previous studies carried out in MRS broth helped to evaluate how different fermentation parameters could influence and improve the production of GABA by *L. plantarum K16.* Thereafter, different agri-food by-products such as tomato, green pepper, apple, and orange pulp and seeds (obtained from private suppliers) were selected to be used as fermentation substrates to produce GABA (these agri-food by-products were obtained and treated following general guidelines and legislation for experiments carry out with plants). Table 1 shows the main nutritional composition such as carbohydrates, total sugars, protein, fat, amino acids, or minerals of the tomato, green pepper, orange, and apple obtained from European Food Information Resource[42]. The fermentation media from agri-food by-products were firstly prepared by grinding and re-suspending independently 5 g of each by-product into distilled water by stirring. Due to the importance of glucose and yeast extract in *L. plantarum K16* to produce GABA, the media was enriched with extra 25 g/L of glucose and 12 g/L of yeast extract. Subsequently, the pH was adjusted to 5.5 and the medium sterilised by autoclaving[43]. After the sterilisation, these agri-food by-product media were supplied with 500 mM of the precursor MSG, inoculated with 1.2% of *L. plantarum K16* strain and incubated at 34 °C during 96 h. As before, analytical samples of the fermented medium were taken to determine the pH, GABA amount and the CFU/mL.

GABA analysis by UHPLC-MS. An ACQUITY UPLC H-class system (Waters., Milford, MA, USA) with a HILIC column (130 Å pore size; 1.7 µm particle size; 2.1 mm internal diameter; 100 mm length) (Waters) coupled with a SecurityGuard ULTRA Cartridge pre-column (Waters) was used for the analysis of GABA in the different fermented medium samples. Column temperature was set to 30 °C, sample temperature was set to 10 °C, and injection volume was 3 µL. An isocratic elution with a mixed in volume of 5% of acetonitrile (HPLC grade, Scharlab, Barcelona, Spain) and 95% of 0.1% formic acid (LC–MS grade, Scharlab,) prepared in Milli-Q water as mobile phase, and a flow rate of 0.25 mL/min, was used. A triple quadrupole MS equipped with an orthogonal electrospray ionisation (ESI) source (ACQUITY TQD, Waters) was used for detection. The instrument was operated in positive mode electrospray (ESI +), MS settings were used as follows: capillary voltage 3.05 kV, desolvation temperature 400 °C, source temperature 120 °C, cone and desolvation gas (nitrogen) flow 60 L/h and 800 L/h, respectively, and collision gas (argon) flow 0.10 mL/min. High purity nitrogen and argon were used (Nippon Gases, Madrid, Spain). MS was run in multiple reaction monitoring (MRM) including two ion transi-

Composition	Tomato	Apple	Orange	Green pepper
Total carbohydrates	3990	11,400	8900	1600
Total sugars	3350	10,350	8880	1530
Total fat	190	360	200	800
Total protein	950	310	870	630
Amino acids				
Alanine	16	11	25	25
Aspartic acid	103	7	99	89
Arginine	13	6	63	30
Proline	19	6	17	27
Isoleucine	25	6	17	20
Leucine	12	13	28	32
Valine	18	12	29	26
Glutamic acid	335	25	57	82
Minerals				
Calcium	12	6	41	11
Magnesium	11	6	15	10
Potassium	248	120	165	120
Sodium	4	1	1	4
Phosphorus	33	9	5	Nd
Iron	0.2	0.6	0.5	0.5
Selenium	Traces	Traces	Traces	1
Zinc	0.1	0.1	0.2	0.1

Table 1. Nutritional composition (mg/100 g) of tomato, apple, orange, and green pepper by-products[35].

tions for GABA: m/z 104 > 87 for quantification and m/z 104 > 69 for identification. Data acquisition and quantification were performed using MassLynx software version 4.1 (Waters). Quantification was performed against a linear (1/x weighted) regression curve based on duplicate injections of calibration GABA standard solutions.

Statistical analysis. The statistical analysis was carried out using the IBM-SPSS statistics software version 25.0 (IBM, New York USA). One-way analysis of variance (ANOVA) was used to evaluate the presence of statistically significant differences in the amount of GABA produced and the growth of *L. plantarum K16* strain among the fermented media within each fermentation parameter studied. Bonferroni's method was applied for pairwise comparison, and statistical significance was declared at $P \leq 0.05$. In addition, Rho Spearman correlation coefficient was calculated to investigate the relationship between the amount of GABA produced and the nutritional composition of each agri-food by-product used.

Results

Effect of fermentation parameters in the production of GABA using MRS broth. *Percentage of inoculum.* Different initial inoculum percentage, 0.8% (7.41 ± 0.07 log CFU/mL), 1.0% (7.44 ± 0.06 log CFU/mL), 1.2% (7.50 ± 0.03 log CFU/mL) and 1.4% (7.60 ± 0.08 log CFU/mL), were assessed to determine the suitable concentration for producing the greatest GABA. The results, represented in Table 2, show that 977.03 ± 22.08 mg/L of GABA were produced when 0.8% of inoculum was added to the medium, and no significant difference (P > 0.05) was observed with respect to the amount of GABA produced with 1% of inoculum. Likewise, the microbial growth was not significantly different between both inoculum percentages after 96 h of fermentation (Table 2). Nevertheless, using a 1.2% inoculum, the GABA production significantly increased (P ≤ 0.05) to 1419.93 ± 57.47 mg/L, along with a pH of 4.30 ± 0.16 and a microbial growth of 7.31 ± 0.41 log CFU/mL. A higher inoculum, 1.4%, did not significantly (P > 0.05) increase the amount of GABA produced compared with the concentration reached with 1.2%. Consequently, an inoculum of 1.2% was selected to carry out the following experiments.

Initial pH. Several initial pH, between 4.0 and 6.0, was studied, focusing on identifying the most suitable to enhance the GABA synthesis. In this case, after 96 h of fermentation, a concentration of 197.5 ± 11.92 mg/L of GABA and no changes in the pH medium were observed using an initial pH of 4.0 (Table 2). However, when the initial pH raised to 4.5, the GABA amount significantly increased ($P \leq 0.05$) up to 951.05 ± 49.26 mg/L, together with a slight decrease in the media pH up to 4.0. Furthermore, a significant increase ($P \leq 0.05$) in the amount of GABA was obtained when the initial pH was 5.5 reaching the maximum value of GABA produced (1419.93 ± 57.47 mg/L). Contrarily, when the initial pH raised to 6.0, a substantial decrease ($P \leq 0.05$) in the amount of GABA (1323.01 ± 72.08 mg/L) was observed compared with the value observed when the initial pH was 5.5. At the same time, the increase of GABA concentration during 96 h of fermentation was accompanied by

	GABA (mg/L)	Viable counts (log CFU/mL)	pH
Inoculum (percentage-log CFU/mL)			
0.8–7.41	977.03 ± 22.08^{b}	7.11 ± 0.03^{b}	4.46 ± 0.03^{a}
1.0–7.44	1000.23 ± 70.82^{b}	6.99 ± 0.03^{b}	4.42 ± 0.01^{a}
1.2–7.5	1419.93 ± 57.47^{a}	7.31 ± 0.14^{a}	4.30 ± 0.16^{a}
1.4–7.6	1428.27 ± 5.38^{a}	6.83 ± 0.04^{b}	4.42 ± 0.01^{a}
Initial pH			
4.0	197.50 ± 11.92^{d}	8.07 ± 0.01^{a}	3.97 ± 0.02^{b}
4.5	951.05 ± 49.26^{c}	6.68 ± 0.03^{c}	4.03 ± 0.01^{b}
5.5	1419.93 ± 57.47^{a}	7.31 ± 0.14^{b}	4.30 ± 0.16^{a}
6.0	1323.01 ± 72.08^{b}	7.91 ± 0.03^{a}	4.21 ± 0.01^{a}
MSG (mM)			
100	174.17 ± 46.7^{d}	6.90 ± 0.11^{b}	3.64 ± 0.01^{b}
300	1207.14 ± 60.38^{b}	7.43 ± 0.05^{a}	4.05 ± 0.02^{b}
500	1419.93 ± 57.47^{a}	7.31 ± 0.14^{a}	4.30 ± 0.16^{a}
550	1027.81 ± 38.21^{c}	7.39 ± 0.04^{a}	4.36 ± 0.02^{a}
Glucose (g/L)			
20	896.4 ± 29.85^{d}	7.37 ± 0.02^{a}	4.43 ± 0.03^{a}
23	1391.2 ± 64.84^{c}	7.29 ± 0.03^{a}	4.45 ± 0.01^{a}
25	2115.7 ± 73.83^{a}	7.40 ± 0.14^{a}	4.43 ± 0.02^{a}
27	1771.6 ± 63.61^{b}	7.28 ± 0.02^{a}	4.37 ± 0.02^{a}

Table 2. GABA (mg/L), viable counts (log CFU/mL) and pH values obtained with *Lactiplantibacillus plantarum K16* in MRS broth, after 96 h of fermentation, using different percentages of inoculum, initial pH, MSG, and glucose concentration (Different letter superscripts indicate if the results are statistically significant ($P \leq 0.05$) in the GABA, viable counts or pH values among the levels of each fermentation parameter).

a decrease in the growth of *L. plantarum K16* strain, hitting the concentration of 7.31 ± 0.14 log CFU/mL when the initial pH was 5.5.

Concentration of MSG. The increase of MSG concentration showed a significant improve ($P \leq 0.05$) in the GABA yield by *L. plantarum K16* strain (Table 2). Specifically, an MSG concentration of 100 mM resulted in 174.17 ± 46.7 mg/L of GABA and a microbial growth of 6.90 ± 0.11 log CFU/mL, and the amount of GABA significantly increased ($P \leq 0.05$) up to 1207.14 ± 60.38 mg/L when the concentration of MSG was 300 mM. The maximum GABA production (1419.93 ± 57.47 mg/L) was reached at 500 mM of MSG concentration, although a significant decrease ($P \leq 0.05$) in the amount of GABA was observed at MSG concentration greater than 500 mM (1027.81 ± 38.21 mg/L). On the other hand, no significant variation ($P > 0.05$) in the microbial growth was observed when the MSG concentration was higher than 300 mM (Table 2).

Concentration of glucose. Glucose concentrations from 20 to 27 g/L were used to test the impact of this sugar on GABA production by *L. plantarum K16* strain. In the media with 20 g/L of glucose the concentration of GABA was 896.4 ± 29.85 mg/L and the microbial cell growth was 7.37 ± 0.02 log CFU/mL (Table 2). A significant increase ($P \leq 0.05$) of GABA synthesis (1391 ± 64.84 mg/L) was observed when the glucose concentration reached 23 g/L in the medium. The maximum concentration of GABA (2115.70 ± 73.83 mg/L) was observed with 25 g/L of glucose, but a higher concentration of glucose (27 g/L) resulted in a significant decrease ($P \leq 0.05$) in the amount of GABA produced (1771.6 ± 63.61 mg/L) (Table 2). Regardless of the concentration of glucose supplied to the culture medium, the microbial cell growth did not significantly change ($P > 0.05$) maintaining viable counts around 7 log CFU/mL (Table 2). According with these results, 25 g/L of glucose supplementation was considered the optimal concentration to obtain the highest GABA amount during fermentation.

GABA production using agri-food by-products. A production trial of GABA was performed using different kinds of agri-food by-products as fermentation substrates for *L. plantarum K16*. In this case, GABA synthesis was stimulated by applying the best conditions observed using MRS broth. Therefore, the GABA produced in MRS broth was considered the control and was used to compare the results observed in the fermented by-products. The results show that the fermentation of apple by-product yielded 1166.81 ± 27.46 mg/L of GABA and a microbial cell growth of 8.13 ± 0.04 log CFU/mL (Table 3). GABA production using orange by-product was quite similar (1280.01 ± 59.22 mg/L) to that of apple by-product but with a significant increase ($P \leq 0.05$) in the microbial growth reaching a concentration of 8.88 ± 0.14 log CFU/mL. Green pepper and tomato by-products significantly ($P \leq 0.05$) enhanced the biosynthetic pathway of GABA producing 1626.52 ± 55.9 mg/L and 1776.75 ± 109.49 mg/L, respectively (Table 3). However, the GABA yield of *L. plantarum K16* was significantly higher (2115.7 mg/L) compared to the values observed using agri-food by-products.

Agri-food by-product	GABA (mg/L)	Viable counts (log CFU/mL)	pH
Control (MRS broth)	2115.7 ± 73.83^{a}	7.40 ± 0.14^{c}	4.43 ± 0.02^{a}
Tomato	1776.75 ± 109.49^{b}	8.17 ± 0.02^{b}	4.44 ± 0.01^{a}
Green pepper	1626.52 ± 55.90^{b}	7.69 ± 0.08^{c}	4.46 ± 0.01^{a}
Apple	1166.81 ± 27.46^{c}	8.13 ± 0.04^{b}	4.27 ± 0.01^{b}
Orange	1280.01 ± 53.22^{c}	8.88 ± 0.14^{a}	4.29 ± 0.04^{b}

Table 3. Content of GABA (mg/L), viable counts (log CFU/mL) and pH values achieved with *L. plantarum K16* fermenting tomato, green pepper, apple, and orange by-products and MRS broth as a control (Different letter superscripts indicate if the results are statistically significant ($P \leq 0.05$) in the GABA content, viable counts, or pH values among the agri-food by-products).

Discussion

The first aim of this research has focused on identifying fermentation parameters involved in GABA synthesis. Therefore, an OFAT experiment was carried out in MRS broth to evaluate how the percentage of inoculum, initial pH, MSG, and glucose concentration influence *L. plantarum K16* to produce GABA. When the percentage of inoculum was assayed, a significant increase in the amount of GABA was observed using an inoculum of 7.5 log CFU/mL (1.2%) compared to using an inoculum of 7.41 log CFU/mL (0.8%) (Table 2). However, an inoculum of 7.6 log CFU/mL (1.4%) did not significantly increase GABA yield. Other studies also reported the importance of the inoculum concentration to enhance the biosynthesis of GABA. For instance, Kantachote et al.[40] showed that *L. plantarum DW12* produced the highest concentration of GABA (128 mg/L) when the initial inoculum was 7 log CFU/mL giving a microbial cell growth of 8.01 log CFU/mL. However, a higher inoculum (8 log CFU/mL) increased the microbial cell growth to 9.2 log CFU/mL but yielded 101 mg/L of GABA. Rayavarapu et al.[28] showed that the highest amount of GABA produced by *L. fermentum* was 3.79 g/L and the microbial cell growth was 5.8 log CFU/mL using a 1% of inoculum. However, an increase of inoculum to 2% did not significantly change the GABA production yielding 3.71 g/L and a microbial cell growth of 6.4 log CUF/mL. Even lower GABA synthesis was observed when the inoculum used was 3 or 4% obtaining 2.62 and 2.12 g/L of GABA, respectively.

Regarding the initial pH of the culture medium, LAB are broadly adapted to a wide range of pH values mainly due to the acid stress caused by their metabolism, because LAB normally produce a wide amount of lactic acid from carbohydrates fermentation. A high concentration of lactic acid creates a stressful environment in the medium that could negatively influence bacterial development and cause growth inhibition, while nutrients are still available, as well as increase cell death[44]. Consequently, LAB have developed protective mechanisms to avoid cell damage[45]. In this sense, Heunis et al.[46] identified about 300 proteins involved in the protection of *L. plantarum 423* strain against acid stress. Most of protective mechanisms try to maintain the intracellular pH using proton pumps, decarboxylation, deamination, metabolism changes, or strengthening the cell envelop. Fernández and Zúñiga[47] highlighted the importance of the catabolism of amino acids, such as aspartic acid, arginine or glutamic acid, as critical coping mechanism to overcome stressful environments. The GAD pathway is considered one of the most essential acid tolerance systems, which is based on the decarboxylation of glutamic acid by a GAD enzyme resulting in a molecule of GABA, classified as an alkaline compound[48]. In addition, during GAD pathway, a cytoplasmic proton is consumed increasing the internal pH and improving cell homeostasis maintenance[49]. Shin et al.[50] indicated that the catalytic activity of GAD enzyme is extremely dependent on pH, and the optimum pH value significantly enhance the relative activity of the enzyme and thus the GABA yield. In our study, the highest GABA production (Table 2) was detected when the initial pH was 5.5. Zhang et al.[51] and Chen et al.[41] also reported that other *L. plantarum* strains produced the highest amount of GABA in MRS broth when the initial pH of the medium was 5.5. Similarly, Tanamool et al.[52] reported that an increase in the initial pH from 4.0 to 6.0 significantly increased the amount of GABA produced (from 2 to 14 g/L) by a *L. plantarum* strain isolated from fermented fish products.

Generally, LAB are considered nutritionally fastidious microorganisms, which need the supplementation of vitamins and amino acids required for a proper metabolism performance[53]. Hence, the development of *L. plantarum* strains could be linked to the supplementation of amino acids because, in many cases, these bacteria are unable to produce these compounds. For instance, *L. plantarum* could need L-Glu supplementation to metabolise it and enhance the bacteria growth[54]. Likewise, L-Glu could also be required to activate the secondary metabolism to produce postbiotic compounds such as GABA[55] or plantaricin[56]. Furthermore, L-Glu is usually supplemented directly into the fermentation media of *L. plantarum* strains due to this amino acid is the GABA precursor[57]. In the same way, MSG has been used in several studies to enhance GABA synthesis[58–60]. However, the MSG concentration should be optimised for each strain due to an excessive MSG concentration could be toxic and suppress the GAD enzyme[55]. In this investigation, increasing the concentration of MSG from 100 to 500 mM significantly enhanced GABA synthesis, but a reduction in GABA production was observed by supplying 550 mM of MSG (Table 2). Harnentis et al.[26] also reported that *L. plantarum N5,* isolated from buffalo milk, achieved the highest amount of GABA (18 g/L) using a glutamate concentration of 500 mM. However, since MSG concentration is strain-dependent, other studies performed with *L. plantarum* strains reported that 80 and 200 mM of MSG were optimal for GABA synthesis[61,62]. Similarly, Yogeswara et al.[58] studied the GABA production of *L. plantarum FNCC 260* strain using a wide range of MSG concentrations. The results showed a maximum GABA production (1226 mg/L) by supplying to MRS broth with 100 mM of MSG. Gomaa[63] required a concentration of 750 mM MSG to get the maximum GABA yield (14.5 g/L) using *L. plantarum DSM749* strain isolated from Egyptian dairy

products. Among other LAB species, the optimum amount of MSG can be also highly variable. Villegas et al.[64] studied the GABA production using an *L. brevis* strain isolated from quinoa sourdough. MRS medium was supplied with concentrations of MSG up to 400 mM, reaching the highest concentration of GABA (26.29 g/L) with 270 mM of MSG. Likewise, Wu et al.[30] increased the efficiency of the GABA synthesis by *L. brevis RK03* strain reaching 62.53 mg/L of GABA by supplying 650 mM of MSG to the fermented medium.

The source of sugar is also essential for LAB species to produce energy and cell biomass[65]. In this regard, glucose is considered the most attractive carbohydrate commonly used to enhance bacterial cell growth and lactic acid production[66,67]. Moreover, glucose catabolism produces severe acidification of the medium that could trigger the activation of the GAD pathway and thus, the stimulation of GABA synthesis[68]. In the present study, when the MRS broth contained 25 g/L of glucose, *L. plantarum K16* synthetised the great concentration of 2115.7 mg/L of GABA. Furthermore, Hussin et al.[69] reported the highest GABA synthesis by *L. plantarum Taj-Apis362* using 20 g/L of glucose. However, *L. plantarum EJ2014* only required 10 g/L of glucose to yield 19.8 g/L of GABA[70] and *L. plantarum KCTC3103* showed the maximum GABA production (670 mg/L) using 5 g/L of glucose[71]. Contrary, Zareian et al.[72] using *L. plantarum MNZ* strain isolated from fermented soybean showed the highest GABA (408.36 mg/L) biosynthesis when 60 g/L of glucose were supplied to the fermentation media.

The fermentation process in MRS broth helped identify the essential parameters to produce GABA by *L. plantarum K16*. The maximum concentration of GABA (2115.7 mg/L) was obtained using MRS broth composed of 25 g/L of glucose, 12 g/L of yeast extract, 500 mM of MSG, an initial pH of 5.5, an inoculum of 1.2%, incubated at 34 °C and fermented for 96 h. Therefore, after identifying the best conditions to produce the maximum amount of GABA by *L. plantarum K16* in MRS broth, a fermentation trial was performed to assess the ability of this bacteria to produce GABA in agri-food by-products. According to the nutritional and functional value of orange, green pepper, tomato, and apple, their pulp and seeds by-products were considered suitable raw materials for fermentation. Several authors[73,74] have proposed recycling apple waste by using it as a fermentation substrate due to its high concentration of magnesium, calcium, fibre, and phenolic compounds like flavonoids or hydroxycinnamic acid. Moreover, more than half of the raw material from the orange juice industry are wasted, which means the loss of a good source of dietary fibre, phenolic compounds, and minerals[75,76]. Likewise, pepper and tomato by-products are also considered good sources of dietary fibre, phenolic compounds, proteins, carbohydrates, and lipids[77,78]. Likewise, Table 1 shows that apple by-products had the highest concentration of total carbohydrates and sugars, followed by orange, green pepper, and tomato. However, the protein content in apple by-product was the lowest compared with that of tomato by-product. Furthermore, the tomato by-product reported the highest concentration of L-Glu (335 mg/100 g), followed by green pepper, orange, and apple by-product. Despite the nutritional variability between these four by-products, in the present study, they were enriched with 25 g/L of glucose, 12 g/L of yeast extract and 500 mM of MSG, to ensure that at least *L. plantarum K16* had enough nutrients to synthetise GABA. Furthermore, *L. plantarum K16* produced great amount of GABA reaching a concentration of 1166.81 mg/L, 1280.01 mg/L, 1626.52 mg/L and 1776.75 mg/L in apple, orange, green, and tomato by-products, respectively (Table 3). However, the GABA produced using MRS broth was significantly higher than the concentration obtained with any of the agri-food by-products. Sharma et al.[79] also evaluated if *L. plantarum LP-9* could produce GABA using saccharified agro-residues such as wheat rice, corn bran or cassava. In this case, they also performed a previous optimisation process, in MRS broth, of relevant parameters for GABA production, such as MSG, pH, and temperature. Then, these optimised parameters were applied in those agri-residues showing the maximum production of GABA (1.39 g/L) using cassava, but it was lower concentration than the one observed in MRS broth (1.53 g/L). Contrarily, Moo-Chang et al.[80] showed that *L. sakei B2-16* in MRS broth enriched with 4% of sucrose, 1% of yeast extract and 5% of MSG (conditions previously optimised) could produce 28.05 g/L of GABA. However, significantly higher concentration of GABA, 68.05 g/L, was obtained using the by-product rice bran extract enriched with 4% of sucrose, 1%yeast extract and 12% of MSG.

In the present study, the difference in GABA yield between each agri-food by-product could be related to the variability in their nutritional composition. Regarding Table 1, carbohydrate and sugar concentrations of the agri-food by-products were inversely correlated ($\geq|0.6|$) with GABA production. However, the microbial cell growth showed a positive correlation (≥ 0.4) with carbohydrate and sugar content. On the other hand, it was observed a strong direct correlation (≥ 0.8) between the content of GABA and protein, as well as the concentration of L-Glu (≥ 0.9). This could mean that agri-food by-products with high content of sugar and carbohydrates could enhance metabolic pathways involved in cell duplication. Nevertheless, a higher protein and L-Glu concentration could enhance the GAD pathway.

Furthermore, the different production of GABA, between MRS broth and by-products, could be due to agri-food by-products present a wide and great variety of compounds compared to MRS broth, which composition is fully controlled. Thus, the variability of compounds in each agri-food by-product could have different effects on *L. plantarum K16* metabolism. For example, several compounds could activate other metabolic pathways on *L. plantarum K16* strain by focusing more on these biochemical processes than on the GAD pathway. Several studies have reported the importance of other metabolic routes that protect LAB under stressful conditions such as arginine or agmatine deaminase pathways or aspartic acid or histidine decarboxylation processes[44,47–82]. Therefore, after confirmed that tomato, orange, apple, and green pepper by-products could be used to produce GABA by *L. plantarum K16*. Further research is necessary to characterise the composition of each by-product and design a specific optimisation process for each by-product to maximise the GABA production of *L. plantarum K16*.

Conclusions

A wide range of relevant parameters involved in the GABA production were individually studied to achieve the highest yield of *L. plantarum K16* strain. The optimisation of the percentage of inoculum, the initial pH, MSG, and glucose concentration, strongly influenced the GAD pathway of *L. plantarum K16* and significantly

increased the GABA production in MRS broth. Afterwards, GABA production was successfully achieved using tomato, green pepper, apple, and orange by-products by applying previously optimised fermentation parameters.

Data availability

All the data generated in the study are included in the present manuscript. All the materials described are available from the corresponding author upon reasonable request.

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Author contributions

M.C.H. and L.D.G. performed the literature review, design the experiments, carried out the experiments, evaluation of raw data and statistical analysis. L.S.V., A.E. and J.S. collaborate in the investigation process. L.J.R.B. collaborate in the evaluation of data and statistical analysis. M.C.H., L.J.R.B. and L.D.G. were a major contributor in writing the manuscript. L.S.V., A.E. and J.S. helped on Writing—Review & Editing. All authors read and approved the final manuscript.

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Additional information

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ANNEX II.I: ADDITIONAL MATERIAL

Chapter 27

The role of probiotics in nutritional health: probiotics as nutribiotics

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27.1 Nutribiotics: ways to improve the nutritional status

In recent years the intestinal microbiota has been of great interest since it is involved in many functions in humans and animals (Kraimi et al., 2019). This intestinal microbiota is composed of a wide variety of microorganisms, such as bacteria, Archaea, viruses, and eukaryotes (including protozoa and fungi). For years, scientific studies carried out in this field have shown that the intestinal microbiota influences the immune function, having a strong impact on health (Alverdy & Luo, 2017; O'Mahony et al., 2014; Sampson et al., 2016; Williams et al., 2011). Likewise, gut microbiota performs a fundamental role in the control of homeostatic processes, such as nutrient metabolism or micronutrient synthesis. For this reason, the deterioration of the intestinal microbiota can cause an imbalance known as dysbiosis, and thus many intestinal diseases could be triggered due to inadequate homeostatic regulation (Cammarota et al., 2014). Consequently, the imbalance in the intestinal microbiota facilitates the generation of many pathological states that involve infections with pathogens or metabolic disorders (Alverdy & Luo, 2017; O'Mahony et al., 2014; Sampson et al., 2016; Williams et al., 2011). Furthermore, the intestinal microbiota also plays a very important role in many extraintestinal tissues and in various development and metabolism processes in organs, such as the liver, adipose tissue, and bone (Sommer & Bäckhed, 2013).

Moreover, evidence has shown that the balance of the intestinal microbiota could be restored using live microorganisms, known as probiotics, which when administered in adequate amounts confer a benefit for the host's health (FAO/WHO, 2006). For instance, Korpela et al. (2018) showed the beneficial effect of commercial probiotics, such as *Bifidobacterium breve* or *Lactobacillus rhamnosus*, conducting a study with infants who were likely to develop allergic diseases due to their microbiota disruption after using antibiotics.

Probiotics can also aid in the homeostasis preservation through the modulation of the immune system with the regulation of immunoglobulins and cytokines, the stimulation of macrophages, and the response against food antigens. As well as, probiotics can reinforce the intestinal epithelial barrier, promote nutrients absorption or enhance the proliferation of other beneficial microorganisms, and inhibit other pathogens (Sehrawat et al., 2020). Therefore these beneficial effects of probiotics may help in the prevention or treatment of diseases related to the gastrointestinal (GI) tract, alterations in the immune system, hepatic diseases, neoplastic proliferation, the cardiovascular system, or intolerances, among others (Brown & Valiere, 2004).

Due to the wide variety of potential health benefits of probiotics, Arora and Baldi (2015) proposed to split the classification of probiotics into pharmabiotics and nutribiotics. Considering pharmabiotics those microorganisms used to treat or prevent medical illnesses by giving physiological and pharmacological benefits. However, the concept of nutribiotics would be focused more on treating nutritional problems, enhancing the benefits of food or dietary supplements, and preserving human health. Hence, these authors indicated that the term nutribiotics would embrace the probiotic microorganisms, and the products obtained from these microorganisms with specific nutritional claims, currently known as postbiotics Fig. 27.1.

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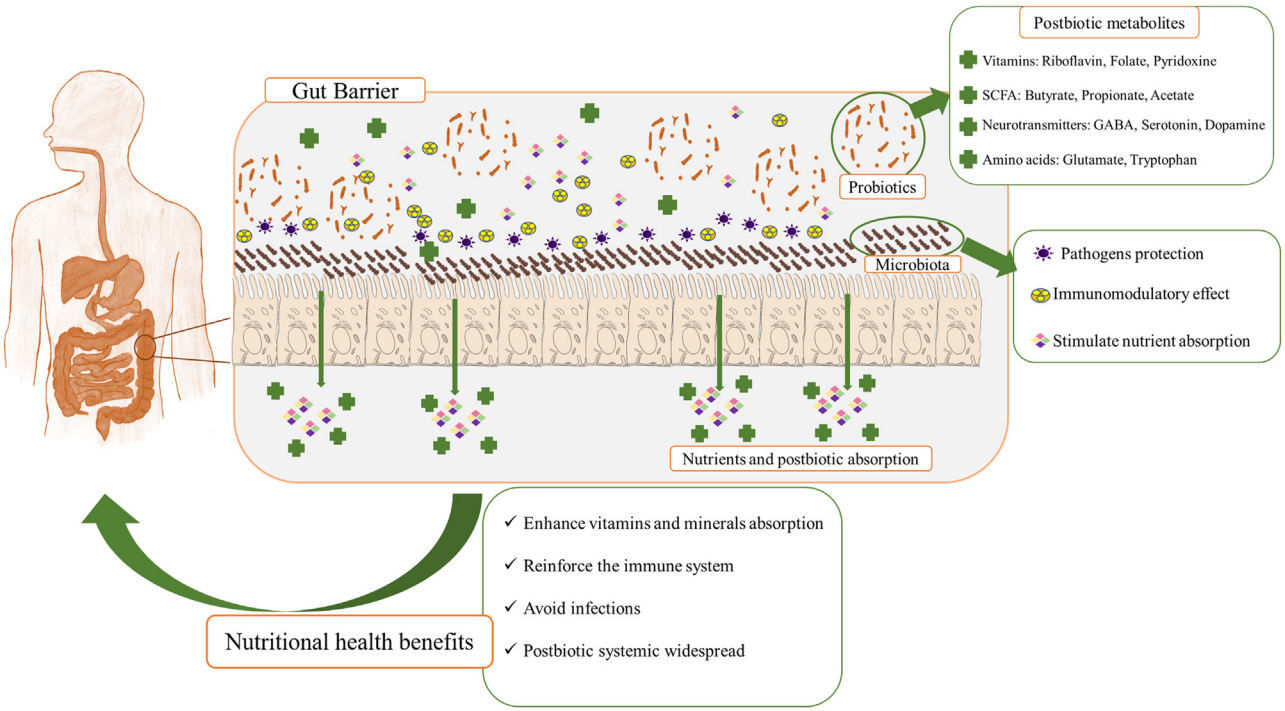

FIGURE 27.1 Overview of gut microbiota and probiotics health benefits. The figure shows how the probiotics in the intestinal microbiota produce postbiotics that are metabolites, such as vitamins, short-chain fatty acids, neurotransmitters, and amino acids. Probiotics and postbiotics allow maintaining a healthy intestinal barrier by generating protection against pathogens, as well as having an immunomodulatory effect and stimulating the absorption of nutrients. This combination allows healthy effects through nutrition through the intake of these compounds (probiotics and postbiotics). *From Diez-Gutiérrez L., San Vicente L., R. Barrón L.J., Villarán, M. del C. and Chávarri M., Gamma-aminobutyric acid and probiotics: Multiple health benefits and their future in the global functional food and nutraceuticals market, Journal of Functional Foods 64, 2020, 1–14. https://doi.org/10.1016/j.jff.2019.103669. Image edited by the author Lucía Diez-Gutiérrez.*

27.1.1 Probiotics: source, variety, and potential

Conventionally, probiotic microorganism has been isolated from dairy products, such as different types of cheeses, kefir, buttermilk and yogurt, and human GI tract or breast milk. According to the current demand of probiotics, the screening of these microorganisms has moved to unconventional raw materials focusing on traditional fermented foods, fruits, vegetables, or other natural sources (Sornplang & Piyadeatsoontorn, 2016). For instance, Yu et al. (2013) used traditional Chinese sauerkraut to isolate probiotics and identified their potential beneficial properties. Erginkaya et al. (2018) performed a similar isolation process of probiotic strains from traditional Turkish dairy products, for instance, Tulum cheese, yogurt, cokelek, camis cream, and kefir.

Currently, the variety of probiotics is mainly composed of lactic acid bacteria (LAB) that are classified as Gram-positive cocci or bacilli cytochrome and catalase-negative characterized by producing a large amount of lactic acid during the fermentation of sugars. This kind of microorganisms are also classified as nonspore-forming and aerotolerant, which could admit a low concentration of oxygen, or microaerophilic, or need a lack of oxygen, anaerobic. The LAB group is mostly composed by bacteria from genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Weisella*, *Pediococcus*, and *Oenococcus* (Papadimitriou et al., 2016; Yépez & Tenea, 2015). Other probiotics closely linked to LAB are the *Bifidobacterium* genera. These bacteria are anaerobic gram-positive curved and bifurcated rod-shaped, which are generally classified as catalase negative and nonspore-forming (Shah, 2011). According to the sugar fermentation, *Bifidobacterium* strains also produce lactic acid but mainly produce acetic acid. Moreover, *Bifidobacterium* genome encodes the fructose-6-phosphate phosphoketolase that catalyzes the breakdown of hexose phosphate molecules into erythrose-4-phosphate plus acetyl phosphate. However, this pathway is not present in LAB being therefore a suitable test to distinguish both groups (Hoover, 2014).

Lee et al. (2018) made an overview of the *Lactobacillus* strains (*L. acidophilus*, *L. fermentum*, *L. reuteri*, or *L. plantarum*), *Bifidobacterium* strains (*Bifidobacterium bifidum*, *Bifidobacterium breve*, or *Bifidobacterium animalis*), or *Streptococcus thermophilus* that have been currently classified as nutribiotics.

Furthermore, several studies have reported that some yeasts, such as *Saccharomyces cerevisiae*, *D. hansenii*, *Kluyveromyces lactis*, *Yarrowia lipolitica*, or *Torulaspora delbrueckii*, could have the potential to be classified as probiotics. Nevertheless, none of these yeasts have been properly classified as probiotics (Hatoum et al., 2012). Palma et al. (2015) explained that *Saccharomyces boulardi*, belonging to the same species as *S. cerevisiae*, is the only yeast currently declared as a human probiotic, proved by different clinical trials. For a long time, probiotic activity research has mainly focused on bacteria but ongoing studies are trying to prove the probiotic effect of different types of yeasts and fungi (Chuang et al., 2020). For instance, Wang et al. (2019) studied the potential probiotic effect of the nonpathogenic yeast *Diutina rugosa*, which has resistance against GI environment and the ability to colonize and adhere to intestinal cells, considered proper probiotic characteristics. Karim et al. (2020) has reported that *Kluyveromyces marxianus* is a promising nonconventional probiotic yeast due to its potential health benefits, such as immune system and cholesterol modulation, antioxidative properties and resistance against adverse GI conditions. Moreover, the fungus *Aspergillus oryzae* also present potential probiotic effects, such as prevention of bacterial infection, potential to reconstruct the microbiota, or maintenance of the immune system, due to the studies performed with pigs, poultries, and fishes (Dawood et al., 2020). The same happens with *Eurotium cristatus (Aspergillus cristatus*), which can modulate the gut microbiota of mice (Kang et al., 2019).

Despite the wide variety of potential probiotic microorganisms, specific characteristics are required to be considered as probiotics. Fontana et al. (2013) portrayed that probiotics should be Generally Regarded as Safe microorganisms for instance, they cannot present any pathogenic, toxic, or another potential side negative effect. In addition, they indicated that probiotics need to survive adverse environment conditions related to the GI tract, such as the high concentration of bile salts and the pH stress. FAO/WHO (2006) reported specific safety and functionality properties that need to be assayed in vitro, such as hemolytic activity, resistance against pH and salinity, microorganism aggregation, antibiotic resistance, antimicrobial activity, adhesion ability to gut cells, cholesterol modulation, or immunomodulatory effect. Angmo et al. (2016) reported that the resistance against acidic pH, lysozyme, and bile salts were relevant characteristics to survive the stressful environment of the GI tract. Moreover, they reported that aggregation and hydrophobicity are properties related to the ability of microorganisms to adhere to intestinal epithelial cells. In addition, these authors highlighted the importance of the characterization of probiotics without potential harmful properties related to hemolytic activity, pathogen inhibitory effect, or antibiotic resistance capacity.

27.1.2 Postbiotics: bioactive probiotic products

Nowadays, probiotic research is moving toward the importance of probiotic products due to their potential beneficial effect on health. These probiotic products have been defined as postbiotics, including metabolic compounds and the nonviable bacterial products that present a relevant biological activity (George et al., 2018). Cuevas-González et al. (2020) have even specified postbiotics are considered only soluble factors produced by bacteria metabolism or released after the probiotic breakdown. In this regard, they considered that dead probiotics are best classified as paraprobiotics.

Furthermore, among the metabolic compounds considered as postbiotics are organic acids, lipids, proteins, and other complex molecules, which can modulate the immune system, inflammatory response, cholesterol accumulation, or antioxidant effect (Aguilar-Toalá et al., 2018). Diez-Gutiérrez et al. (2020) summarized different compounds considered postbiotics due to their potential health benefit, such as short-chain fatty acids (SCFA) (acetate, butyrate, or propionate), vitamins (folate, biotin, or riboflavin), bacteriocins (nisin or glycocin), neurotransmitters [gamma-aminobutyric acid (GABA), serotonin, dopamine, or acetylcholine], or mediators of inflammation (lactocepin).

A good way to determine the ability of probiotics to produce postbiotic metabolites is using cell-free supernatants (CFS) obtained after probiotic in vitro fermentation under specific conditions. Thus supernatants obtained from probiotics, such as *L. acidophilus* and *L. casei*, could modulate the inflammatory response decreasing the TNF-α secretion and increasing the synthesis of IL-10 in the intestinal epithelia (Żółkiewicz et al., 2020). Moreover, Moradi et al. (2019) studied the potential benefits of CFS produced by *Lactobacillus* strains, such as *L. acidophilus*, *L. casei*, and *L. salivarius*. The characterization was based on the analysis of the antimicrobial effect of the lyophilized CFS against the pathogen *L. monocytogenes*, the influence of CFS in the formation of biofilms, and the potential cytotoxic effect. Promising results were shown for CFS produced by *L. salivarius* reporting high effectiveness against *L. monocytogenes*, resistance under stressful environments, and safe for consumption.

Likewise, researchers have analyzed the potential applications of postbiotics improving probiotics efficiency or, even, use postbiotics as active ingredients due to their potency against several types of diseases (Hernández-Granados & Franco-Robles, 2020). Singh et al. (2018) gather information about the beneficial implications of postbiotics. Their highlighted postbiotics effects, such as the modulation of neural diseases, alterations in the immune system, metabolic disorders, cardiovascular diseases, or pathogen infections.

27.2 Nutritional health benefits of probiotics and postbiotics

The wide variety of beneficial effects from probiotic supplementation could be reinforced with postbiotics, as they could enhance the crosstalk between the host and the gut microbiota. According to the huge amount of probiotic microorganisms and the substances included in the postbiotic term, diverse mechanisms of action are expected to undertake their beneficial effect on the gut microbiota (Żółkiewicz et al., 2020). Million et al. (2017) supported the relationship between the alteration of the gut microbiota and malnutrition situations. Malnutrition is considered a worldwide concern that affects millions of people every year mainly produced by low dietary intake, malabsorption of micro- or macronutrients or higher energy requirements. The malnutrition term includes the range of under- and overnutrition situations. Likewise, malnutrition situations could be related to other types of health problems worsening the symptomatology and decreasing the quality of life. For instance, malnutrition could be associated to gastroenterology illnesses (Norman et al., 2006), cancer (de Pinho et al., 2019), or infectious diseases (Rai et al., 2002). The potential beneficial effects that probiotics could have under several nutritional health disorders are shown in Table 27.1.

27.2.1 Undernutrition situations

Undernutrition embraces the deficit of several macronutrients, such as proteins, and micronutrients like vitamins and minerals. Generally, this kind of deficiencies produce the disruption of the body homeostasis and triggers health problems associated with skin alterations, liver disturbances, or diarrhea associated to the imbalanced microbiota (Million et al., 2017). Therefore several research have wonder how probiotics could improve the nutritional status of malnourished people. Sheridan et al. (2014) conducted an in-depth review of the most common malnutrition situations and the potential of probiotics to address these concerns in susceptible population, such as children, pregnant women, or elderly people.

27.2.1.1 Children nutritional deficiencies

Kambale et al. (2021) reported that around 200 million children are currently suffering undernutrition and this situation supposes the 45% of children death every year. They consider that diarrhea is one of the biggest problems as it leads to infections, increase the situation of severe malnutrition and raise the death rate. Therefore several studies have focused on severe acute malnutrition (SAM) due to its high incidence and the severe side effects produced, such as weak immune system, cognitive deficiencies or appearance of the nutritional edema called kwashiorkor. Also, this nutritional problem could enhance the development of other types of diseases such diabetes, coronary problems, pneumonia and infections produced by *S. aureus*, *Salmonella*, *Klebsiella*, or *E. coli* (Million et al., 2017; Sheridan et al., 2014). Kerac et al. (2009) performed the PRONUT study based on a randomized double-blind placebo-controlled trial with 795 children who suffered SAM and used a combination of four different well-known probiotics, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *Lactobacillus paracasei*, and *Lactobacillus plantarum*, combined with several prebiotics. The results of this study suggested that probiotics have the potential to improve the health of SAM children and decrease the mortality rate. Following this study, Grenov et al. (2017) developed another double-blind, randomized, placebo-controlled study with 400 SAM children and they evaluated how the probiotics *B. animalis* and *L. rhamnosus* influenced diarrhea and pneumonia caused by SAM. Likewise, the results showed that probiotics could reduce the time of suffering severe diarrhea and decrease the mortality rate. Castro-Mejía et al. (2020) also evaluated the probiotic effect of *L. rhamnosus* and *B. animalis* in SAM children. In this case, they analyzed the evolution of the gut microbiota when these probiotics were administered. The consumption of probiotics decreases the days with diarrhea associated to SAM; however, heterogenic results were shown if they presented kwashiorkor or not.

Following this trend, Alou et al. (2017) went one step further using metagenomic and culturomic techniques to accurately identify the microorganisms involved in SAM. For that purpose, the microbiota of SAM and healthy children were investigated to determine the deficient microorganisms, which could be characterized and potentially supplied as probiotics. The results showed a wide variety of potential probiotics, such as *Bacillus subtilis*, *B. adolescentis*, *Weisella confusa*, or *L. parabuchneri*, which presented different functions in the microbiota, such as antioxidant activity, antibacterial function, mutualism with other microorganisms, or production of postbiotics.

More recently, Kambale et al. (2021) carried out a systematic review of the trials performed since 1990 to 2020 in people affected by SAM. They indicated that gut microbiota alteration, mainly *B. longum* absence, affects the synthesis of vitamins, energy harvest, or immune system development, which is linked to malabsorption, pathogens' infection, and diarrhea. As well, the diarrhea decreases the absorption of proteins, potassium, zinc, or other micronutrients essentials for the correct body function. The probiotic supplementation increases the absorption of calcium, zinc, or different

TABLE 27.1 Representation of the probiotics that present a nutritional health benefit.

Nutritional health		Probiotic	Probiotic health	References
Undernutrition	Severe acute malnutrition in children	*L. paracasei*, *L. plantarum*, *L. rhamnosus*, *B. animalis*, *B. adolescentis*, *W. confusa*, *P. pentosaceus*, *B. subtilis*	• Alleviate diarrhea • Increase vitamins and minerals absorption • Synthetize of B group vitamins	Grenov et al. (2017), Kerac et al. (2009), and Leblanc et al. (2011)
	Pregnancy malnourishment	*L. rhamnosus*, *B. lactis*, *L. acidophilus*, *L. plantarum*, *B. animalis*, *L. fermentum*, *L. reuteri*, *S. thermophilus*	• Enhance iron assimilation • Stimulate folate production and metabolisms	Ballini et al. (2020) and Rusu et al. (2020)
	Frailty syndrome in the elderly	*L. reuteri*, *B. longum*, *L. helveticus*, *L. paracasei*, *L. plantarum*, *L. brevis*, *L. zymae*, *B. bifidum*, *L. bulgaricus*	• Increase vitamin D synthesis • Modulate inflammatory response • Synthetize neurotransmitters as GABA serotonin or dopamine	Diez-Gutiérrez et al. (2020), Lei et al. (2016), and Rizzoli and Biver (2020)
Overnutrition	Cardiovascular diseases	*Enterococcus* sp., *L. plantarum*	• Cholesterol modulation • Increase bile salts elimination	Liu et al. (2017) and Nuhwa et al. (2019)
	Metabolic disorders	*L. rhamnosus*, *L. plantarum*, *L. gasseri*, *L. acidophilus*, *L. curvatus*, *B. breve*	• Modulate glucose levels • Interfere in adipocytes functionality	Cani and Van Hul (2015) and Mallappa et al. (2012)
Malnutrition associated to other disorders	Irritable bowel diseases	*L. rhamnosus*, *L. plantarum*, *L. bulgaricus*, *B. animalis*, *B. longum*, *S. boulardi*, *L. reuteri*, *L. fermentum*	• Increase micronutrients absorption • Modulate the inflammatory response • Enhance the synthesis of amino acids and SCFA	Lee et al. (2018), Lichtenstein et al. (2016), Martínez-Abad et al. (2016), and Turroni et al. (2012)
	Pathogens infection	*L. reuteri*, *L. acidophilus*, *L. bulgaricus*, *S. thermophilus*, *L. plantarum*, *S. faecium*	• Enhance the immune system • Reinforce mucosal barrier • Increase the nutritional status avoiding longer illnesses	Goderska et al. (2018) and Ruggiero (2014)
	Food intolerances	*B. lactis*, *L. casei*, *B. longum*, *L. acidophilus*, *S. thermophilus*, *B. infantis*	• Protect against intestinal cell damage • Increase nutrients absorption	Gingold-Belfer et al. (2020) and Sousa Moraes et al. (2014)

vitamins and avoids pathogens' colonization. Hence, probiotics' potential leads to alleviate diarrhea, decrease the risk to develop anemia, and reduce hospitalization time. Moreover, probiotics could also alleviate SAM through the synthesis of useful postbiotic compounds, such as vitamins or bacteriocins. Leblanc et al. (2011) indicated the potential of several LAB to produce B group vitamins, such as riboflavin, folate, or vitamin B12.

27.2.1.2 Pregnant women nutritional deficiencies

Maternal and fetal health could be compromised due to the deficiency of micronutrients, such as vitamins and minerals, which are necessary to preserve the body homeostasis, cell functionality, and metabolic activity. Therefore these micronutrients have an essential influence on pregnancy through the materno-placental fetal axis. For instance, vitamins' deficiency in pregnant women could affect the organogenesis process or could trigger epigenetic disturbances due to the alteration in the DNA methylation leading to an increased likelihood that the fetus will develop insulin resistance, obesity, or hypertension (Gernand et al., 2016). Mantaring et al. (2018) reported that pregnant and breastfeeding women need to consume supplements, such as iron, folate, or vitamin B6, to enhance the proper development of the baby. Hence, this study was focused on assessing the beneficial effect of using probiotics combined with maternal nutritional supplements. Promising results were obtained after the oral consumption of *L. rhamnosus* and *B. lactis* combined with different kind of vitamins, minerals, proteins, and lipids due to the increase of the nutrient absorption and the energy density. Furthermore, Bisanz et al. (2015) used a yogurt fortified with the probiotic *L. rhamnosus* combined with Moringa plant characterized due to its high amount of vitamin A, iron, zinc, or calcium, among others. This combination could be a cheap way to preserve a healthy microbiota and avoid the micronutrients deficiency. Khalili et al. (2020) studied how to increase the folate content into yogurt by adding different types of probiotics, such as *S. thermophilus*, *B. lactis*, *L. acidophilus*, or *L. plantarum*, that could produce folate as a postbiotic. The results obtained showed a higher amount of folate with the fortification with *L. plantarum* strains, suggesting that some probiotics could be an alternative way to the synthetic folic acid. Following this trend, Bardosono et al. (2019) performed a clinical trial with pregnant women to evaluate how the probiotic *B. animalis* could modulate the plasma levels of vitamins B12, B6, and folate. This research showed that the supplementation of this probiotic increased the blood levels of these vitamins providing potential health benefits. For example, vitamin B12 enhances the metabolism of folate and decreases the risk of cardiovascular diseases (CAD) avoiding the synthesis of homocysteine and the combination of this vitamin with B6 and folate maintains the correct methylation process involved in the synthesis of DNA and thus in the development of the fetus. Ballini et al. (2020) carried out a pilot study with 20 pregnant women to determine the effect of a probiotic mix and the kiwi fruit powder in the availability of folate. The probiotics used in this experiment, *B. infantis*, *L. plantarum*, *L. rhamnosus*, *L. fermentum*, *L. reuteri*, and *L. acidophilus*, were selected according to their ability to increase the absorption of nutrients, the natural synthesis of folate, and the modulation of the immune system. Likewise, the consumption of these probiotics supposed an increase in the folate concentration coupled with the modulation of the levels of sugar in blood and the women weight during the gestational time.

Anemia is another health problem commonly associated to pregnant women who suffered micronutrients deficiency, and it is experienced by about half of pregnant women worldwide. Generally, this health problem is produced by the deficiency of vitamin A, considered essential for the embryogenesis process, cell maintenance, tissue synthesis or hematopoiesis, or iron needed for the immune system and neurological maintenance (Van Den Broek, 2003). Vonderheid et al. (2019) performed a wide analysis to determine how probiotics could increase the absorption of iron. A significant increase in the iron absorption was reported with the supplementation of *L. plantarum* strains. Rusu et al. (2020) also carried out a wide research of the most significant probiotics that could alleviate anemia through the increase in iron absorption and bioavailability. In this review, they highlighted the potential effect of *L. fermentum*, which could interact with enterocytes to improve the iron levels and enhance its absorption, *L. acidophilus*, involved in the ferritin formation and iron assimilation, or *S. thermophilus*, that influence the iron binding, hemoglobin and ferritin.

27.2.1.3 Elderly nutritional deficiencies

During the last decades, the ageing process has been directly linked to the modification of the gut microbiota through the decrease of beneficial microorganisms coupled with an increase of potentially pathogenic bacteria. Consequently, elderly people could suffer the deterioration of some essential biological functions required for a healthy microbiota, such as low levels of SCFA or reduction of macro and micronutrients absorption, which leads to malnutrition situations (Salazar et al., 2017). Poor nutritional status in elderly people could produce the frailty syndrome (FS) supposing the loss of organs functionality, increase the DNA damage, or enhance metabolic disorders (Salvatella-Flores & Bermúdez-Humarán, 2020). Lorenzo-López et al. (2017) evaluated the relation between FS and the nutritional status. They

highlighted that the development of this syndrome is related to the low consumption and assimilation of essential micronutrients, such as vitamin B6, D, E, or C, folate, or macronutrients including proteins and thus amino acids. Salazar et al. (2017) agreed on the relationship between the deficiency of some vitamins, proteins, and iron and added that this kind of alterations could trigger neurological disorders, anorexia, loss of bone mass, depletion of the immune system, or gut alterations. Recently, Davinelli et al. (2021) supported that the improvement of the nutritional status using functional nutrients could decrease the likelihood to suffer FS. Based on their review, they considered functional nutrients those involved in specific physiological benefits including mineral and vitamins supplements, carotenoids, prebiotics, and probiotics. Patel et al. (2014) explained that probiotics could enhance the solubility of minerals like calcium and magnesium through the synthesis of SCFA, such as butyrate and lactate, which increase the absorption of these compounds and thus promote bone health. Rizzoli and Biver (2020) also supported the potential of probiotics to maintain the health of bone. For instance, probiotics, such as *L. reuteri*, *L. paracasei*, *B. longum* and *L. helveticus*, could reduce the osteoclastic bone resorption decreasing the response of proinflammatory cytokines. Likewise, those probiotics could also increase the levels of vitamin D by producing lactic acid, which indirectly stimulates the expression of vitamin D receptors. Lei et al. (2016) carried out a clinical trial with 417 elderly people who had suffered a distal radius fracture. In this case, *L. casei* was used to determine the effect of this microorganisms on the patient's recovery. The results indicated that the consumption of probiotics could decrease the recovery time.

Moreover, the FS has also been linked to cognitive deterioration associated to different types of dementia, Alzheimer's disease, and Parkinson's disease. The improvement of the nutritional status with the supplementation of antioxidants, flavonoids, and vitamins C, B, E, or D may prevent or delay the worsening of these diseases (Gómez-Gómez & Zapico, 2019). Also, scientific evidence currently remarks the important connection between the gut and brain, how the gut microbiota influences the development of neurological diseases and the phsychomodulatory effect of probiotic microorganisms. Therefore probiotic strains that can produce positive psychiatric effects on patients with psychopathologies are defined as psychobiotics (Tyagi et al., 2020). This type of probiotics influences the relation between the host and the brain exerting antidepressant effects that can alter emotional, cognitive, and neuronal indices (Dinan et al., 2013). As mentioned before, psychobiotics act by reducing host neurodegeneration by decreasing oxidative stress, modulating cytokine milieu and thus reducing circulating proinflammatory cytokines and/or altering brain hormones or neurotrophic factors. In addition, psychobiotic action is strain and species specific, as well as the mechanism of action with respect to reduction of mental stress (Talbott et al., 2019). For example, Lister (2020) analyzed the effect of nutrition and lifestyle on the development of Parkinson disease. This study suggested that nutritional supplements, such as vitamins B and D, probiotics, antioxidants, or flavonoids, could decrease the inflammation process. Szczechowiak et al. (2019) summarized the impact diet and nutritional status in the progression of Alzheimer disease, indicating the antiinflammatory effect of vitamins complexes, probiotics, flavonoids like resveratrol, polyphenols like curcumins, or alkaloids as caffeine. Diez-Gutiérrez et al. (2020) reported that postbiotics, such as GABA, serotonin, dopamine, or acetylcholine, could have a beneficial effect of neurological disorders.

27.2.2 Overnutrition situations

Overnutrition is considered another type of malnutrition associated to the excessive consumption of nutrients. This exaggerated nutrient intake produces the mitochondria and endoplasmic reticulum stress due to the high concentration of metabolites that need to be processed and assimilated. Therefore the imbalance between nutrient intake and the energy consumed have a side effect on the proper function of the enzymes involved in catabolism and triggers the stimulation of other enzymes which generates a metabolic imbalance. Also, this nutrient overload leads to the increased accumulation of fat or lipogenesis. Currently, overnutritions situations enhance the provability to develop cardiometabolic disorders, such as hypertension, diabetes, metabolic disorders, or obesity (Aggarwal et al., 2012; Qiu & Schlegel, 2018).

27.2.2.1 Cardiovascular diseases

CVD are an increasing cause of death generally caused by smoking, obesity, a sedentary routine, diabetes, stress, or lipid abnormalities. According to this information, alteration in the levels of body lipids, such as low- and high-density lipoprotein-cholesterol, LDL-C and HDLC-C, or triglycerides, could increase the likelihood to develop CVD (Thushara et al., 2016). DiRienzo (2014) explained that high levels of LDL-C could trigger coronary heart disease (CHD) through the formation of atherosclerotic plaques. Therefore therapies have been developed to decrease the risk to develop CHD focusing on lowering the LDL-C. In addition, low levels of HDL-C or high levels of triacylglycerol and

triglyceride-rich proteins could increase the risk to develop CHD. Likewise, Nuhwa et al. (2019) reported how LABs isolated from flowers can modulate the cholesterol levels by testing the capacity of these LABs to assimilate cholesterol. The results showed that seven *Enterococcus* sp. and four *L. plantarum* presented bile salt hydrolase (BSH) activity coupled with high cholesterol assimilation. In addition, the assimilation of cholesterol and the BSH activity suggests that these probiotics are promising ways to prevent hypercholesterolemia diseases. Liu et al. (2017) performed an in vivo study to determine the ability of *L. plantarum* strainsto modulate the levels of cholesterol. Positive results were obtained because this probiotic reduced the liver and serum cholesterol, regulated the levels of triglycerides, and increased the bile acids elimination. Hence, *L. plantarum* could be considered as a tool to prevent CVD.

27.2.2.2 Metabolic disorders

The metabolic syndrome (Ms) is a worldwide concern mainly produced in obese people. This pathology could increase the risk to develop CVD, raise the blood pressure, or trigger insulin resistance (Grundy, 2016). Mallappa et al. (2012) presented the potential benefits of using probiotics in patients who suffered metabolic disorders, such as obesity, diabetes, and Ms. Probiotics, such as *L. rhamnosus*, *L. plantarum*, *Lactobacillus gasseri*, or *L. acidophilus*, were highlighted due to their ability to reduce the cholesterol, modulate adipocytes functionality, and thus maintain the body weight avoiding the fat accumulation. Cani and Van Hul (2015) added that *B. animalis*, *B. breve*, *L. curvatus*, or *L. reuteri* could have important metabolic effects through the modulation of glucose levels, cholesterol, and triglycerides concentration. Also, these probiotic could modulate the inflammatory response, decrease the accumulation of fats in the liver and preserve the body weight, essential characteristics to prevent Ms, diabetes, and obesity. The systematic review of Tenorio-Jiménez et al. (2020) analyzed the effect of probiotics in clinical trials performed in Ms patients. Beneficial effects were shown with the supplementation of probiotics and indicated that these microorganisms could be used as a good adjuvant to current therapies.

27.2.2.3 Malnutrition and other health disorders

27.2.2.3.1 Gastrointestinal disorders

For a long time, probiotics have been used as a complement to treat GI tract disorders. Disorders mainly produced by dysbiosis or other alterations in the microbiota that can affect the correct functioning of the GI tract. Generally, the available probiotics present interesting mechanisms which involve the regulation of inflammatory cascades, absorption of nutrients, modulation of hypersensitivity reactions, improvement of the GI barrier, and suppression of pathogens. Therefore Lee et al. (2018) explained that some probiotics, such as *L. rhamnosus*, *L. plantarum*, *L. bulgaricus*, or *B. animalis*, have been used to treat different nutritional problems that involved disorders, such as antibiotic-associated diarrhea, acute diarrhea, or food intolerances, among others. Hence, there is a wide variety of investigations that were conducted to test the effect of these probiotics in several GI disorders (Verna & Lucak, 2010).

Brown and Mullin (2011) supported that patients with irritable bowel disease (IBD), such as Crohn's disease (CD), or ulcerative colitis (UC), required specific dietary guidelines to maintain their life quality. Among these guidelines, they remarked the importance of consuming supplements, such as multivitamins, minerals, probiotics, or prebiotics, trying to avoid any nutritional deficiency. Judkins et al. (2020) remarked the importance of using probiotics IBD patients to enhance the permeability and nutrient absorption decreasing the malnutrition risk and improving the immune system.

For example, *B. longum* is considered the most common *Bifidobacterium* species found in the GI tract of adults and infants (Turroni et al., 2012). Palma et al. (2015) reported a lower level of *B. longum* in the stool of CD patients than in healthy individuals. In this sense, the oral administration of *B. longum* could provide beneficial effects on human health (Zhang et al., 2019). In the same way, Tamaki et al. (2016) evaluated the efficiency of *B. longum* in UC patients. The trial showed that the supplementation of this probiotic could modulate the production of cytokines and enhance the mucosal barrier, suggesting that this microorganism could be a promising complement for UC patients.

Furthermore, Lichtenstein et al. (2016) summarized how probiotic therapies could alleviate Crohn's patients and they highlighted that *S. boulardi* could be a promising probiotic for this disease compared to other probiotic microorganisms. Fedorak et al. (2015) also evaluated the benefits of probiotics in Crohn's patients. In this case, they found out that the supplementation of single strain probiotic was not significantly beneficial for patients. However, the consumption of the mixture called VSL#3, composed of four strains of *Lactobacillus*, three strains of *Bifidobacterium*, and a strain of *Streptococcus salivarius*, could be useful to decrease the inflammatory response.

Similarly, Martínez-Abad et al. (2016) focused on the immunomodulatory effect of the probiotics *L. rhamnosus*, *L. fermentum*, and *B. lactis*. Their mechanisms of action could be helpful to modulate the immune response of IBD patients. Similar modulation of the immune response in patients affected by IBD was shown by *L. plantarum* strains.

Also, the consumption of this probiotic could alleviate the symptomatology IBD, such as abdominal bloating and pain (Vries et al., 2006).

Studies have also focus on the potential effect of probiotic and postbiotics combination against GI disorders. Haileselassie et al. (2016) evaluated how immune cells responded after the supplementation of CFS rich in postbiotics produced by *L. reuteri*. These postbiotics had a strong effect on the regulation of dendritic cells followed by the influence on regulatory T cells. Moreover, an increase in the synthesis of IL-10 was shown along with a reduction in the expression of genes related to proinflammatory response. These results indicated that the identification of the postbiotics presented in the CFS was needed to use them in clinical assays based on necrotizing enterocolitis (NEC) or IBS. In this regard, Patel et al. (2014) concluded that probiotics are a good way to prevent NEC development as these microorganisms can fight against pathogen colonization, strengthen the intestinal epithelial barrier, and block inflammatory pathways. Therefore probiotics can be considered essential for the prevention of NEC and postbiotics could be used to enhance their effectiveness. Butyric acid is a promising postbiotic useful for NEC because this SCFA can suppress the inflammatory response, modulate apoptosis, and maintain the colon cell structure. Russo et al. (2019) also indicated that SCFAs and tryptophan are postbiotics with a potentially positive effect on CD and UC through the interconnection of the gut microbiota with the innate and adaptative immune cells.

27.2.2.3.2 Pathogens infection

Probiotic supplementation has also moved around the infection of the GI tract by *Helicobacter pylori*, characterized due to its high infection rate, which can trigger chronic gastritis, gastric adenocarcinoma, or peptic ulcer. Gonzalez and López-Carrillo (2010) indicated the importance of the nutritional status to decrease the likelihood to develop cancer. Likewise, several studies have been focused on using probiotics as a complementary treatment to this pathogen Goderska et al. (2018). Zhang et al. (2019) explained how *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* strains could be useful to eradicate *H. pylori* as they could inhibit the urease activity, avoid cell adhesion, stimulate the immune system, and reinforce the mucosal barrier.

27.2.2.3.3 Food intolerances

As well as, studies were carried out trying to determine the benefits of probiotics in different types of food intolerances. Sousa Moraes et al. (2014) summarized how probiotic microorganisms could counteract the side effects produced by gluten proteins, such as gliadins and glutenins, which trigger the development of the celiac disease. They indicated that microorganisms, such as *B. lactis*, *L. casei*, and *B. longum*, could protect the epithelial cells against the damage caused by gliadins. Moreover, in this study, they highlighted the effectiveness of using a combination of probiotic strains, such as VSL#3, which hydrolyzes gliadins more efficiently than single-strain probiotics. Gingold-Belfer et al. (2020) performed a clinical trial with lactose-intolerant patients who were treated with a probiotic cocktail called Bio-25 composed by 11 different strains, including *L. acidophilus*, *L. rhamnosus*, *L. casei*, *B. breve*, *S. thermophilus*, *B. longum*, and *B. infantis*. Significant reduction of the symptoms and enhancement of the lactose absorption was shown due to the β-galactosidase activity of the probiotics supplied.

27.3 Encapsulation technology for the development of functional ingredients

As previously stated, intestinal microbiota influences immune functions and the development and metabolism processes in the different organs of body, including the brain. If the alteration of the microbiota leads to a disease development, triggered by an inadequate homeostatic regulation, the challenge is to manage the composition of the microbiota and compensate for any alterations that may occur to minimize the negative impact on the immune functions or metabolism processes in the host.

The restoration of the intestinal microbiota using live microorganisms requires the definition of dietary supplementation strategies that allow these microorganisms to reach the intestine alive (Ma et al., 2019; Roselino et al., 2020). Likewise, if the compounds of interest are postbiotics or parabiotics, the stability of these molecules must be guaranteed in the environment of the intestinal microbiota (Perez-Burgos et al., 2013; Wu et al., 2020). In both postbiotic and parabiotic cases, the formulation and processing of a food or nutritional or nutraceutical supplement can result in a loss of the desired functionality when the probiotic dies or the postbiotic or parabiotic is not functional.

Food matrix composition (pH, nutritional composition, water activity, natural antibiotic presence, etc.) may alter the probiotic cell viability during the processing and storage time, as well as during the GI transit after intake. Dairy products are considered as effective vector for the probiotic bacteria delivery into the GI tract, due to the high buffering

capacity of milk proteins, which can protect the bacterial cells during gastric transit. Aljutaily et al. (2020) evaluated the influence of the food matrix on the mouse gut microbiota enriched with *Clostridium butyricum* used as probiotic. The presence of prebiotics, milk protein with high buffering capacity, or dense structure of dairy products were relevant factors for the bacteria viability. Chocolate is another interesting food matrix to improve the probiotic cell viability. The low water activity of this food matrix linked to the presence of protective substances, such as milk proteins and sugars, contributes to a high stability of probiotic bacteria, such as *Bacillus coagulans*, *Lactobacillus*, or *Bifidobacterium*, during storage (Cielecka-Piontek et al., 2020; Kobus-Cisowska et al., 2019). However, the convenience of other food matrices, such as fruits or vegetable matrices, depends on pH value, concentration of lactic and acetic acids and presence of antioxidant and antimicrobial substances. For example, the fermentation of a tomato juice with different *Lactobacillus* spp. leads to changes in pH, acidity, and sugar content that can affect probiotic viability, and in consequence, limiting the storage time and conditions (Yoon et al., 2004). Therefore the application of probiotic cultures in different food matrices is nowadays a great challenge for the food industry.

In this regard, the encapsulation of probiotics, prebiotics, postbiotics, and parabiotics is probably one of the most promising and successful strategies to achieve the protection of these components until their release in the large intestine. Microencapsulation may be defined as the process of enveloping or surrounding any substance (encapsulated material, in this case, bacteria, yeasts, postbiotics, etc.) within another substance (encapsulating material preferably biopolymers) on a very small scale, yielding microcapsules ranging from less than one micron to several hundred microns in size. The main purpose of microencapsulation is to produce particles that control mass transport behavior in some way. The microcapsule matrix or shell is designed to prevent diffusion of material from or into the microcapsule to achieve the protection of sensitive components in an oxidative o degradative environment. However, at the same time, the encapsulated material must be released in the large intestine and the mechanism should be controlled by pH change, transit time, or colonic microbiota enzymes.

Depending on the nature of encapsulated substances, the purpose of the encapsulation, and the release mechanism selected, different encapsulation technologies should be applied (Chávarri et al., 2012). The first aspect to be taken into account for selecting the encapsulation technology is the structure of the microcapsules that, in turn, will influence their functionality. Encapsulation technologies, such as spray-, freeze-, and vacuum drying and some extraction and coacervation processes generate regular or irregular geometry microcapsules containing small portions of encapsulated material (Fig. 27.2A). This multinuclear structure of the microcapsule allows a slow release of the encapsulated substances as the degradation of the matrix occurs, in comparison to the rapid release that would occur in the rupture of the mononuclear microcapsule obtained by some coacervation or emulsion processes (Fig. 27.2B). However, a well-established core−shell structure could offer a higher stability of the encapsulated active substances because they are not trapped onto the particle surface. Multinuclear microcapsules are usually more easily produced but the wall layer is not equally distributed over the multinuclear structures and it is difficult to achieve a standardization of the release kinetics in a set of these microcapsules as shown in Fig. 27.2 (Chávarri et al., 2012).

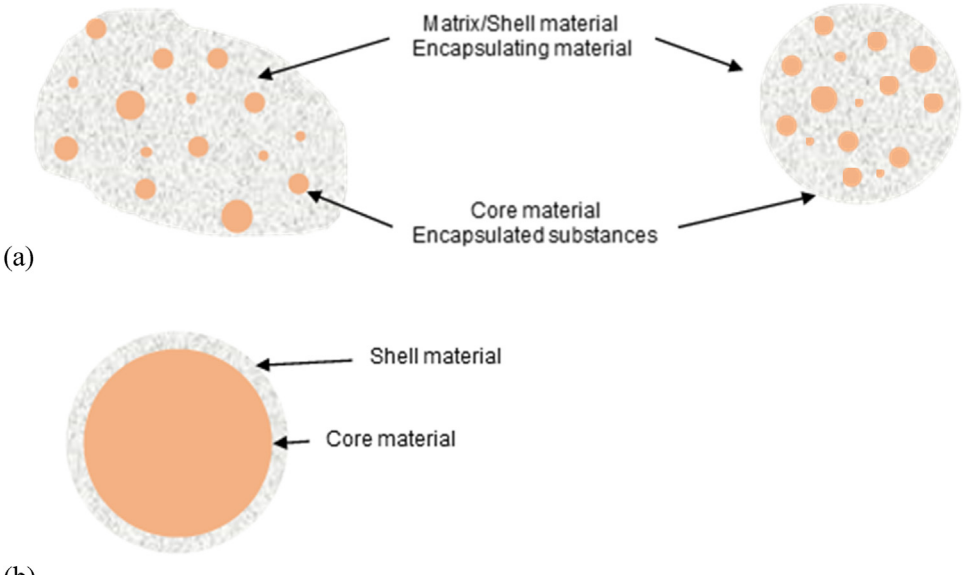

FIGURE 27.2 Multinuclear (A) and mononuclear (B) microcapsule structures. In the figure A, two different structures of microcapsules are observed, the corresponds to the multinuclear structure of a microcapsule where both the encapsulating material and the encapsulated compound are mixed. (B) A mononuclear structure where the center and the shell of the microcapsule are perfectly separated. *From Marañon, I., San Vicente, L., Hidalgo, N., & Chávarri, M., Multilayer probiotic microcapsules. Multilayer Probiotic Microcapsules." (EUROPE No. EP3205216A1). European Patent Application, 2016. Image edited by the author Izaskun Marañón.*

For example, a more cost-effective solution to improve the viability of some probiotic bacteria during food processing and along the product shelf life could be the use of spray-drying or chilling encapsulation technologies. The purpose of these techniques is to extend the survival of the bacteria, using the encapsulating material to isolate bacteria from the food matrix creating a barrier between them. *Lactobacillus acidophilus* and *B. animalis* subsp. *lactis* added to savory cereal bars improved their stability by microencapsulation compared to the incorporation of active or lyophilized bacteria. Encapsulated *Lactobacillus acidiphilus* ($>10^8$ CFU/g) remained stable for 30 days longer than lyophilized form did, until 90 days, and *B. animalis* remained stable for 105 days, 75 days longer than the lyophilized form, because the microorganisms inside the matrix were protected from the environment and remained in a latent state for longer (Bampi et al., 2016). Proper selection of the material of microcapsule shell not only can prolong the shelf life of the probiotic ingredient but also maximize the survival of probiotic cells as they pass through the gastric system. Bustamante et al. (2017) showed that the encapsulating material influenced the bacteria viability but also that this material did not protect in the same way the life of different types of bacteria encapsulated in a multinuclear structure not provided with a continuous shell. In this regard, the use of a combination of maltodextrin with vegetal soluble proteins as encapsulating material in a spray-drying process can improve the *Bifidobacterium infantis* and *Lactobacillus plantarum* viability during storage. Both probiotics showed an increase in resistance to simulated gastric conditions but *L. plantarum* cells were more sensitive to gastric juice than *B. infantis* cells probably due to the cell distribution into the microcapsule structure and the presence of cells on the particle surface.

The use of a three-fluid nozzle for spray-drying has improved the loading capacity and encapsulation efficiency of bioactive compounds due the core−shell droplet formation (Gorgannezhad et al., 2020). Tasch Holkem and Favaro-Trindade (2020) has also used a shell constituted by a mixture of protein complex and polysaccharide to increase the protection of *L. paracasei* and *B. animalis* encapsulated in solid lipid microparticles.

Up to now, the best encapsulation solutions usually require a combination of both types of structures to optimize the efficiency of microencapsulated products by maximizing the amount of active components that reach the large intestine intact. Thus a matrix structure that contains probiotics, postbiotics, parabiotics, or prebiotics covered by a continuous layer of a polymeric material that reduces matrix permeability from the inside to the outside of the microcapsules, or vice versa, can be an interesting solution to achieve functional ingredients more effective. Complex coacervation using chitosan coating on alginate or pectinate beads in which the active component is dispersed is an example of this matrix structure (Chávarri et al., 2012).

In this regard, a double-stage procedure (collection alginate beads from a calcium chloride bath and introducing them into a chitosan bath) to create an alginate-chitosan microcapsule shows a more structured chitosan coating layer (Zaeim, 2020). However, these scientific results require defining profitable and easily scalable processes to be commercially developed. Processes that involve a high number of stages do not usually fit with industrial requirements, so current research is focused on obtaining multilayer structures by applying concentric nozzles. This is the case of the stabilization of probiotics through extrusion in concentric nozzles (Oxley, 2012) or by one-step coaxial electrospinning procedure (Feng et al., 2020). Multilayer structures not only improve the stability of the active substances contained in the microcapsule core but also offer opportunities for more accurate controlled release systems. Furthermore, multilayer structure can achieve serial releases of the different components contained in each of the layers of the microcapsule increasing the dosage effectiveness of the active ingredient (Marañon et al., 2016). Fig. 27.3 depicts a scheme of a three-layer structure where the outer layer is a barrier that protects the inner layers during the gastric transit, the intermediate layer breaks down in the intestine first releasing the prebiotic component, and finally the microcapsule nucleus disintegrates to release a probiotic in the gut. This type of structure can serve for sequential release of different probiotics or for two different release times of the same encapsulated active component as shown in Fig. 27.3 (Oxley, 2012; Zaeim, 2020).

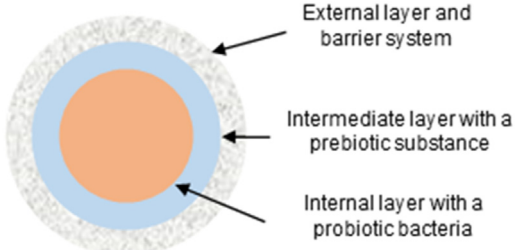

FIGURE 27.3 Multilayer microcapsule scheme. Scheme of a multilayer microcapsule where a probiotic bacterium can be included in the nucleus, in the intermediate layer a prebiotic compound, and in the outermost layer that acts as a barrier. *From Marañon, I., San Vicente, L., Hidalgo, N., & Chávarri, M., Multilayer probiotic microcapsules. Multilayer Probiotic Microcapsules." (EUROPE No. EP3205216A1). European Patent Application, 2016. Image edited by the author Izaskun Marañón.*

On the other hand, multilayer structures offer the possibility of combining separately active ingredients in a single microcapsule that, if found together, their stability would be negatively affected. (Bepeyeva et al., 2017; Chávarri et al., 2010) verified that the encapsulation of *L. gasseri* or *B. bifidum* together with quercetin causes the loss of viability of bacteria during encapsulation process and storage. To improve the bacteria survival, each component of the symbiotic formulation must be individually encapsulated. This procedure involves three individual encapsulation processes and a finished microcapsule mixing process. However, the development of a multilayer structure simplifies the procedure for obtaining the symbiotic. The isolation of quercetin and bacteria in different layers of the same microcapsule allows the symbiotic to develop in an one-stage process. Furthermore, multilayer structures can confine chemically incompatible substances in differentiated layers. In this way, the new microcapsule structure facilitates the dosage for a combination of probiotics, prebiotics, and/or postbiotics (Marañon et al., 2016).

Despite the efforts made to optimize the processes in terms of profitability, the inclusion of new stages in the manufacturing process to stabilize the active components entails a large increase in the total cost for developing of functional ingredients, foods or supplements. The success of encapsulation technologies lies in a greater product efficiency to reduce the dosage of the active components. Consequently, this can lead to a reduction in cost for the manufacturer and at the same time an increase in sales of the product due to greater consumer acceptance.

27.4 Current market of probiotics and future perspectives

The market of probiotic bacteria has experienced significant growth in recent years. Apart from the positive consumer perception due to their healthy properties, prescription of probiotics by healthcare professionals after a surgery or after taking antibiotics is contributing to the increasing consumption of functional foods and supplements containing probiotics.

As a result, and according to the study carried out by Fortune Business Insights (Fortune Business Insights, 2020), the global probiotic market achieved US$ 48.88 billion in 2019 and is expected to reach US$ 94.48 billion in 2027 with a Compound Annual Growth Rate (CAGR) of 7.9% during the forecast period (BCC Research, 2018). These products are becoming more popular for their health benefits and specially for the positive effect on improving the immune response. Last months, as a consequence of coronavirus pandemic, consumers are more conscious of their health and the demand of these products has experimented a notable increase.

According to the study carried out by Market Research Future (BCC Research, 2018), in 2025 the segment with the highest consumption of probiotics will be functional foods and beverages, followed by dietary supplements, animal nutrition and others as shown in Fig. 27.4.

In the food and beverage industry, as well as in dietary supplements and animal feed, the most widely used strains for probiotic ingredients in 2022 will be *Lactobacillus* followed by *Bifidobacterium* and *Streptococcus*. *Lactobacillus* will reach a share of 63%, followed by *Bifidobacterium* reach a share of 27%, *Streptococcus*, and *Bacillus* with shares of 4%, with a CAGR of 8%, 7.8%, and 7.7%, respectively. It is expected that this distribution of markets by bacteria genus will continue in the following years as shown in Fig. 27.5.

Growing consumer concern for health has led to increase demand for healthy food products. Probiotics have proven to have a positive effect on health, especially on digestive health and this makes them products highly demanded by the

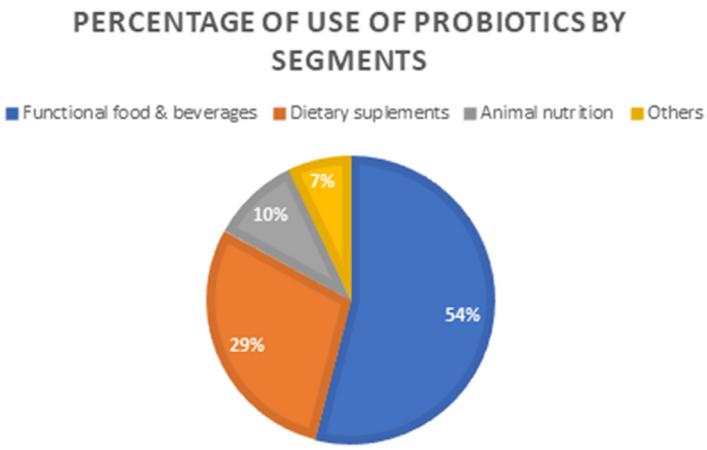

FIGURE 27.4 Distribution of probiotics consumption (%) by segments in 2025. The following figure shows the distribution of probiotic consumption by segments. Specifically, functional foods and beverages have 54%, dietary supplements 29%, animal nutrition 10%, and others 7%. *From Diez-Gutiérrez L., San Vicente L., R. Barrón L.J., Villarán, M. del C. and Chávarri M., Gamma-aminobutyric acid and probiotics: Multiple health benefits and their future in the global functional food and nutraceuticals market, Journal of Functional Foods 64, 2020, 1–14. https://doi.org/10.1016/j.jff.2019.103669. Image edited by the author María del Carmen Villarán.*

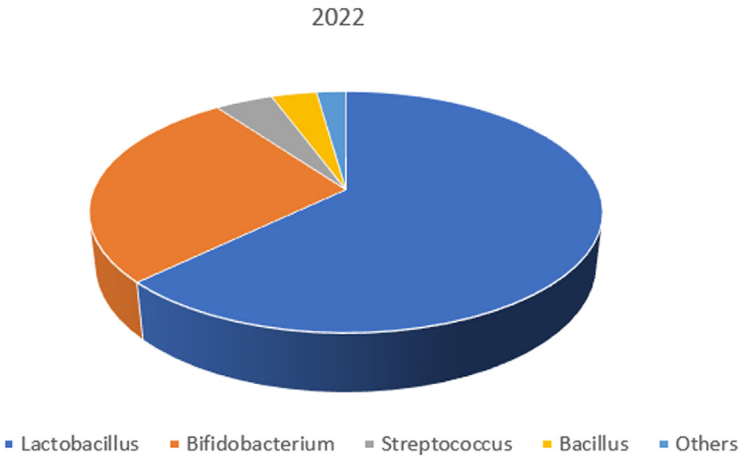

FIGURE 27.5 Global market shares (%) for probiotics foreseen for 2022 (BCC Research, 2018). The following figure represents the world market share in % of probiotics forecast for 2022. Specifically, *Lactobacillus* will reach a share of 63%, followed by *Bifidobacterium* reach a share of 27% and *Streptococcus* and *Bacillus* with shares of 4%. *From Diez-Gutiérrez L., San Vicente L., R. Barrón L.J., Villarán, M. del C. and Chávarri M., Gamma-aminobutyric acid and probiotics: Multiple health benefits and their future in the global functional food and nutraceuticals market, Journal of Functional Foods 64, 2020, 1–14. https://doi.org/10.1016/j.jff.2019.103669. Image edited by the author María del Carmen Villarán.*

population. Consumers look for products that have a proven effect on health and they prefer natural products over drugs. The role of probiotics in the recovery of certain illness is causing the consumer to have high expectations in probiotics as possible products for the prevention of certain diseases.

In addition, probiotics can be incorporated without excessive additional costs into daily products, such as yogurts. Thus they present a low-cost, safe, and natural alternative to drugs for the prevention of certain diseases.

However, the development of the probiotics market and the obtention of new probiotic strains with specific health benefits require significant investment in R&D to respond to several challenges (Binda et al., 2020).

The correct characterization of probiotic strains. It would allow manufacturers to ensure and maintain the purity of their strains and avoid confusions. This complete strain characterization should support their probiotic activity.

Probiotic strains must comply with the safety requirements established by national regulatory entities.

The ability of a probiotics to provide a certain health benefit must be corroborated by at least one human clinical trial performed following recognized guidelines.

The final product, throughout its useful life, must contain the established probiotic dose to provide the declared health effect.

The perception of consumers toward food has changed from products that only provides nutritional value to other products that offer proven health benefits. Among other, consumers recognize the positive effect of probiotic enriched foods on health and on the immune system.

Increased investment by major players in the development of new food products will positively contribute to solve the challenges for new probiotics development with proved claims and to contribute to the increasing the probiotics market.

27.5 Conclusions

Nutribiotics are gaining importance due to their potential beneficial effects to improve the nutritional status of people suffering different disorders associates to malnutrition situations. Several probiotic microorganisms and postbiotics metabolites have been included in this new classification; nevertheless, new investigations could lead to find out new probiotics and postbiotics that could be included in this group. Furthermore, this could suppose an increase in nutribiotics demand to address nutritional problems that could result as even worse health problems.

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ANNEX II.II: ADDITIONAL MATERIAL

Chapter 17

Secondary Metabolites From Probiotic Metabolism

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1 Probiotics

Probiotics are live microorganisms, which when ingested in adequate amounts produce a range of beneficial effects on the health of the host (FAO/WHO, 2006). Probiotics have different biochemical mechanisms to maintain and promote health that include better adhesion to intestinal cells and inhibition of pathogens in these places, improvement of epithelial barrier, regulation of the immune function, and production of antibacterial substances, as well as, postbiotics (Lin et al., 2020). The most common probiotics belong to the genera *Lactobacillus*, which is classified as lactic acid bacteria (LAB), and *Bifidobacterium* (Georgieva, Peikova, Andonova, & Zlatkov, 2014). Within *Lactobacillus* genera, *Lactococcus*, *Enterococcus*, *Streptococcus*, and *Leuconostoc* are also classified as probiotics, as well as some fungi and yeast of genera *Aspergillus* and *Saccharomyces* genera (Amara & Shibl, 2015; Kechagia et al., 2013; Diez-Gutiérrez et al., 2020).

The microorganisms that are used as probiotics are recognized as safe or GRAS, and this safety status may be based either on a history of safe use in food prior to 1958 or on scientific procedures that require the same quantity and quality of evidence, as would be required for a food additive regulation (FDA, 2018). Therefore the term "probiotic" has been related to bacteria with beneficial effects for human health and to which a number of requirements are demanded (Lin et al., 2019), as can see in Table 17.1 (Lin et al., 2020).

Probiotics must be able to survive during their passage through the human gastrointestinal tract (GIT) and subsequently colonize the intestine. In addition, it is necessary that they reach the intestine as viable microbiota and in sufficient amount of approximately 10^7 CFU (Chávarri et al., 2010) in order to provide health benefits. Therefore probiotics must be resistant to the acidic conditions of the stomach and the high concentration of bile acids present in the small intestine (FDA, 2018; Kechagia et al., 2013). Angmo, Kumari, Savitri, & Bhalla, 2016 demonstrated that *Lactobacillus* was able to resist low pH conditions due to the presence of F0F1-ATPase activity (Angmo, Kumari, Savitri, & Bhalla, 2016; Behbahani, Noshad, & Falah, 2019) and that the different susceptibility of LAB to bile acids is due to their hydrolase activity (Angmo, Kumari, Savitri, & Bhalla, 2016; Wang et al., 2016a). In addition, LAB are tolerant to the osmotic pressure necessary for probiotic strains to survive in some foods such as cucumber (Lin et al., 2020). Likewise, Lin et al., 2020 confirmed that probiotic strains were capable of surviving high concentrations of bile acids (0.15%—1.10%), at low pH (2–4) and high osmotic pressures (2%–8%) (Winkelströter, Fabrício, Elaine, & Martinis, 2015; Park & Lim, 2015).

On the other hand, the ability of probiotics to adhere to epithelial cells produces beneficial effects in the intestine. Cell adhesion properties are considered to be correlated to aggregation, coaggregation, and hydrophobicity. In other words, the aggregation and coaggregation properties are important for the adhesion of probiotic strains, since they can direct bacterial adhesion to GIT (autoaggregation) and prevent colonization by pathogenic bacteria (coaggregation) (Armas, Camperio, & Marianelli, 2017). Hydrophobicity is one of the most important factors influencing the strength of bacterial adhesion. Therefore these characteristics allow probiotics to inhibit intestinal adhesion and colonization of pathogenic strains (Behbahani, Noshad, & Falah, 2019) (Table 17.1).

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TABLE 17.1 Requirements demanded for probiotics.

1. Generally Recognized as Safe (GRAS) at the strain level by the United States Food and Drug Administration (FDA) or as qualified presumption of safety (QPS) at the species level by the European Food Safety Authority (EFSA).
2. Obtained from breast milk, gut microbiota, and fermented foods.
3. Long used in food.
4. Proven to be safe, as a food or supplement.
5. A probiotic agent must show nonpathogenic properties.
6. Ability to survive in the digestive tract.
7. Colonization of the intestinal tract.
8. Production of antimicrobial substances.
9. Mainly *Lactobacillus* spp. and *Bifidobacterium* spp.; other bacteria such as *Lactococcus* spp., *Enterococcus* spp., *Streptococcus* spp., *Leuconostoc* spp.; and fungi and yeasts of the genus *Aspergillus* and *Saccharomyces cerevisiae*.

TABLE 17.2 Main functions of probiotics.

1. Produce beneficial metabolites (short-chain fatty acids, bacteriocins, reuterin, linoleic acid, and secondary bile acids).
2. Produce vitamin K and B vitamins [thiamin (B1), riboflavin (B2), pantothenic acid (B5), pyridoxine (B6), biotin (B7), folates (B9), cobalamin (B12)].
3. Produce beneficial proteins/peptides (optimize IgA production, enhance antimicrobial peptides production).
4. Reducing pathogenic toxins.
5. Increase intestinal cell activity and integrity of the epithelial layer.
6. Regulate the immune system and improve the antioxidative system.

Nowadays, one of the important characteristics of probiotics is the safety for human without harboring acquired and transferable antibiotic resistance (Zommiti, Nathalie, Jeannette, & Mounir, 2017). Some probiotic strains with intrinsic antibiotic resistance could be available for restoring the intestinal microbiota after an antibiotic treatment.

In recent decades, researchers have observed that probiotics produce compounds called postbiotics that have a beneficial effect on gut microbiota. Some of these compounds are short-chain fatty acids (SCFAs) that can reduce proinflammatory immune activity (Azad, Kalam, Sarker, Li, & Yin, 2018) as well as improve the integrity of the intestinal epithelial barrier (Park & Lim, 2015; Tulumoğlu, Halil, & Şimşek, 2014), optimize IgA production, modulate homeostatic bile acids production, and increase the production of antimicrobial peptides to prevent pathogen infections (Table 17.2).

Furthermore, probiotics can modulate the intestinal microbiota and generate antioxidant and anticancer compounds that block the synthesis of harmful enzymes in the gut (Molska & Reguła, 2019). Therefore these beneficial microorganisms can act systematically in nutrition, metabolism, physiology, and immunity and assist in the prevention of diseases (Lin et al., 2020).

To date, a large number of scientific articles have been published confirming that probiotics can produce beneficial effects in various gastrointestinal disorders, cardiovascular, and nervous system diseases, among others (Gomi et al., 2018).

2 Postbiotics

Generally, the metabolism is focused on the use of different nutrients that are transformed by biochemical reactions into precursors, known as metabolites, useful for the correct performance of microorganisms (Madigan, Bender, Buckley, & Sattley, 2019). Independently of the microbial species, the primary metabolism is based on the use of nutrients to stimulate cell proliferation and, thus, biomass synthesis (Chubukov, Gerosa, Kochanowski, & Uwe, 2014). Hence, the metabolites involved in primary metabolism are considered the main molecular skeleton of microorganisms (Thirumurugan, Alagappan Cholarajan, Suresh Raja, & Ramasamy, 2018).

By contrast, secondary metabolism plays a completely different role, since it is activated during the late growth phase and the metabolites produced are not essential for basic cell maintenance (Ruiz, Chávez, Forero, & García-Huante, 2010). However, these compounds can act as a defensive line against other organisms, behave as signaling molecules, enhance the transport of other compounds, or serve as bioactive complexes (Marinelli & Marcone, 2011).

According to Thirumurugan, Alagappan Cholarajan, Suresh Raja, & Ramasamy, 2018, more than 2,140,000 secondary metabolites have been described in the scientific literature. In most cases, secondary metabolites are synthesized by plants, followed by bacteria, fungi, and marine organisms such as corals, tunicates, or sponges (Thirumurugan, Alagappan

Cholarajan, Suresh Raja, & Ramasamy, 2018). In the case of microbial metabolites, a wide medical interest has been observed due to their potential therapeutic effect. For instance, several well-known microorganisms such as *Actinomycetes*, *Bacilli*, or probiotic bacteria, such as *Lactobacillus*, *Lactococcus*, or *Bifidobacterium*, can biosynthesize immune suppressants, chemotherapeutic, or antimicrobial compounds useful for human treatments (Craney, Salman, & Nodwell, 2013; Kholia, 2017).

Probiotic secondary metabolites are gaining interest due to the potential beneficial effect they could have in the pharmaceutical and food field (Ruiz, Chávez, Forero, & García-Huante, 2010). Recently, the bioactive functional metabolites from probiotics have been defined as postbiotics (Tsilingiri & Rescigno, 2013). The postbiotic term involves the metabolites or bacteria-free compounds released during the probiotic metabolism that could have a direct or indirect effect in the health of the host (Foo, Loh, Abdul Mutalib, & Abdul Rahim, 2019). Rad, Maleki, Kafil, & Abbasi, 2021 added that postbiotics are also considered those with novel chemical structures, nontoxic effects, and easily absorbed, metabolized, and excreted compounds. The production of this kind of compound is directly linked to specific physicochemical conditions and, therefore, it is possible to focus the metabolism on their synthesis. However, these conditions can also stimulate the synthesis of unknown postbiotics, since their effectiveness have been proven without identification, just considering the supernatant from probiotic fermentation as a postbiotic (Tsilingiri & Rescigno, 2013).

Postbiotic metabolites are gaining interest due to their potential against disease prevention or, even, their treatment. Aguilar-Toalá et al., 2018 highlighted that these metabolic products can modulate blood pressure, inhibit pathogenas colonization, regulate wound healing process, fight against neoplasm development, or increase the antioxidant capacity. Moreover, Foo, Loh, Abdul Mutalib, & Abdul Rahim, 2019 reported the importance of antimicrobial postbiotics and suggested that they could be an interesting substitute for antibiotics. In this sense, this study proved that formulated postbiotic cocktails from *Lactobacillus plantarum* reduced the growth of *Aeromonas hydrophila*, *Enterobacteriaceae*, and other pathogens (Foo, Loh, Abdul Mutalib, & Abdul Rahim, 2019).

Furthermore, postbiotics could be used as an alternative to probiotic supplementation. Despite probiotics are classified as GRAS microorganisms, the ingestion of alive bacteria could trigger undesirable side effects (translocation to other tissues, bacteremia, sepsis, inflammatory response, or resistance genes development) in certain individuals such as young children, immunosuppressed patients, premature neonates, or the elderly (Rad, Maleki, Kafil, & Abbasi, 2021; Wegh, Geerlings, Roeselers, & Belzer, 2019). Tsilingiri et al., 2012 presented the effectiveness of the supernatants obtained from the fermentation of three *Lactobacillus* strains, which were considered the postbiotics, in patients with inflammatory bowel disease. The results showed that the supplementation of probiotics enhanced inflammatory response, however, postbiotics downregulated the inflammatory reaction and conferred protection against *Salmonella* (Tsilingiri et al., 2012).

It is noteworthy that postbiotics could lead to economic savings for food industries compared to probiotics. Probiotics require an important investment to maintain these microorganisms viable and stable to perform the gut colonization after ingestion (Wegh, Geerlings, Roeselers, & Belzer, 2019). On the contrary, postbiotics present a long shelf life up to 5 years, which simplifies the preservation treatments and helps guarantee the quality and food safety (Aguilar-Toalá et al., 2018).

2.1 Postbiotic classification

Postbiotics can be classified according to their molecular nature. The variety of postbiotics can be seen in Fig. 17.1. Amino acids, proteins, vitamins, neurotransmitters, or SCFAs are some of the most relevant postbiotic compounds (Singh, Vishakha, & Singhal, 2018).

2.1.1 Short-chain fatty acids (SCFAs)

Generally, SCFAs are considered to be those carboxylic acids composed by aliphatic tails with a chain length of no more than six carbons. Microorganisms can synthetize these compounds by the anaerobic fermentation of different types of dietary fibers (DF) (Gabriel et al., 2019). Parada Venegas et al., 2019 explained that DF are polymeric carbohydrates nondigestible by the human small intestine. Inulin, resistant starch, pectin, or some types of brans, like oat and wheat, are considered important sources of DF (Parada Venegas et al., 2019). Furthermore, Neis, Cornelis, and Rensen (2015) added that amino acids like glycine (Gly), glutamate, (Glu), threonine (Thr), or aspartate (Asp) could be used as SCFA precursors. For instance, Thr is considered the most significant amino acid for SCFA production due to its ability to be used as a precursor of the three most common SCFAs (Neis et al., 2015).

Among the SCFA group produced by probiotics, acetate, propionate, and butyrate are known as the most influential in human health (Gabriel et al., 2019). *Lactobacillus* species, such as *Lactobacillus buchneri*, *Lactobacillus diolivorans*, or *Lactobacillus reuteri*, are important producers of propionate (Amin, Hashem, Ashour, & Hatti-Kaul, 2013; Zhang, Markus,

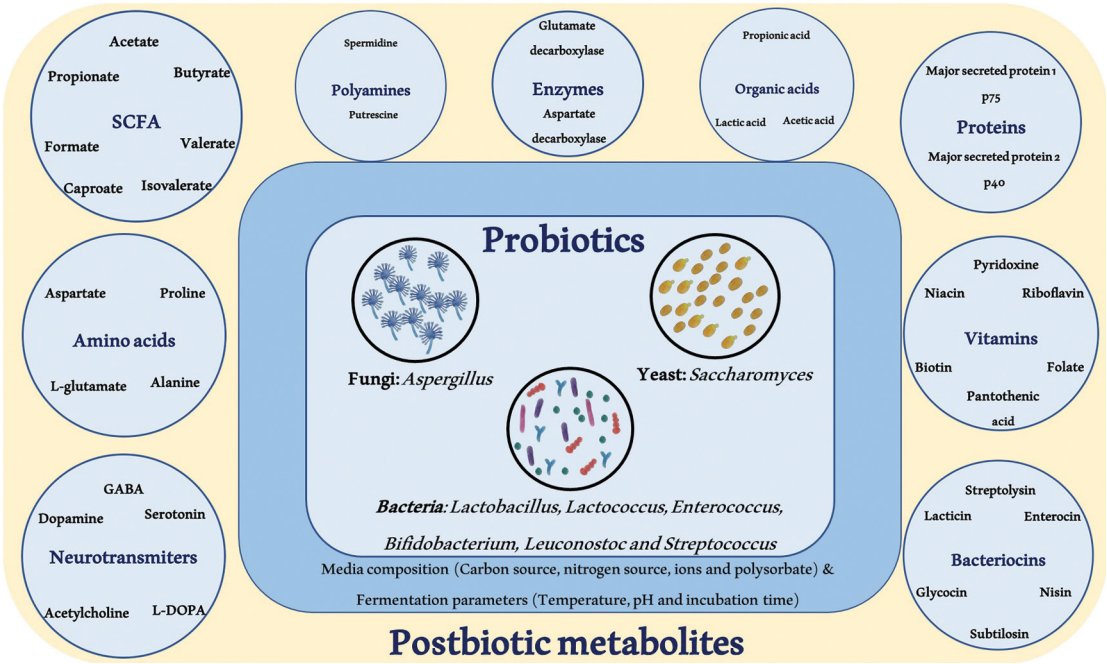

FIGURE 17.1 Summary of the postbiotic metabolites produced by probiotic microorganisms (Singh et al., 2018).

Clarissa, & Michael, 2010), whereas, acetate and butyrate are commonly produced by *Bifidobacterium* species (Parada Venegas et al., 2019).

The biosynthetic process of SCFA begins with the hydrolysis of DF into oligosaccharides and, subsequently, monosaccharides that are converted into the phosphoenolpyruvate (PEP). PEP is common for acetate, butyrate, and propionate biosynthesis. Acetate can be synthesized through the conversion of PEP into pyruvate, which is transformed into acetyl coenzyme A (Acetyl-CoA) and, consequently, Acetyl-CoA is converted into acetate (Ríos-Covián et al., 2016). Likewise, the acetate biosynthesis can be done by using the Wood–Ljungdahl pathway based on the reduction of carbon dioxide into carbon monoxide, which transformation produces Acetyl-CoA and ends in the synthesis of acetate (Parada Venegas et al., 2019).

Besides, propionate can be synthesized by bioconversion of PEP via succinate pathway. However, PEP could be also converted to pyruvate through the acrylate pathway. In this situation, pyruvate is transformed into lactate and its reduction results as propionate (Richards, Li, van Esch, & Garssen, 2016).

Likewise, the biosynthesis of butyrate could be performed via butyrate kinase pathway that is the same as the acetate route till the Acetyl-CoA step (den Besten et al., 2013).

After the production of these three SCFAs, they behave as intestinal protectors with the decreasing of the luminal pH to avoid pathogens colonization (Ríos-Covián et al., 2016). More specifically, butyrate stimulates mucin secretion to block pathogens adhesion. Usually, propionate and acetate are focus on the liver, where they are degraded and used by the hepatocytes (Tan et al., 2014).

2.1.2 Amino acids and proteins

Amino acids can be considered as primary and secondary metabolites because they are intertwined and there is feedback between them (Thirumurugan, Alagappan Cholarajan, Suresh Raja, & Ramasamy, 2018). Among the amino acids classified as essential, probiotics have the ability to synthesize some of them in response to several adverse conditions. Papadimitriou et al., 2016 explained that probiotic like *L. plantarum* or *Lactococcus lactis* can produce Glu, Asp, proline (Pro), and alanine (Ala) in response to osmotic stress. Likewise, amino acids can be biosynthesized against acid stress. Wu, Juan, Guocheng, and Jian (2013) highlighted that *Lactobacillus casei* increases the intracellular accumulation of Asp under low pH, and this amino acid enhances the biomass production of this probiotic.

Some probiotics can go one step further and transformed amino acids into others, which could have different target compared to the precursor amino acids. For instance, after the production of Asp, some *Lactobacilluss,* such as *L. plantarum*, *L. buchneri*, or *Lactobacillus acidophilus*, can increase their resistance against acid environments following the Asp

decarboxylase pathway (AspD). AspD route relieves the acidic stress with the decarboxylation of Asp, as a result, another amino acid is obtained, Ala (Papadimitriou et al., 2016). Other decarboxylase pathways are Glu decarboxylase (GAD) route where Glu is converted into gamma-aminobutyric acid (GABA) or arginine decarboxylation into agmatine (Senouci-Rezkallah, Philippe, & Michel, 2011).

The production of these amino acids as postbiotic compounds could have several beneficial effects in humans because they serve as basic compounds to the synthesis of hormones, neurotransmitters, nucleic acids, or melanin (Aliu, Kanungo, & Arnold, 2018).

Additionally, amino acids can be used by probiotic, such as *L. reuteri* or *L. acidophilus*, to serve as precursors of other beneficial postbiotics. One example is the transformation of tryptophan (Trp) into different indolic acid derivates like indole-3-aldehyde, indole lactic acid, or indole acetic acid (Liu, Alookaran, & Rhoads, 2018). Romani et al., 2014 highlighted the importance of indoles and their antiinflammatory effect, mainly against fungal and yeast infections.

Some research has reported that probiotic bacteria like *Lactobacillus* spp. can even produce full proteins classified as postbiotics. Cicenia et al., 2014 explained that *Lactobacillus rhamnosus* can synthesize two proteins known as p40 and p75 as their molecular mass is around 40 and 75 kDa, respectively. These proteins are considered the first soluble proteins obtained from probiotics. Studies have shown that both proteins can modulate the homeostasis of the intestinal epithelium, inhibit apoptosis, and prevent damage generation in cells and tissues by the tumor necrosis factor (Cicenia et al., 2014).

2.1.3 Neurotransmitters

Neurotransmitters are considered essential chemical compounds in humans since they regulate the neural signaling to ensure the proper body–brain homeostasis (Mora, Segovia, de Blas, & Del Arco, 2012). Surprisingly, some of the most important neurotransmitters can be synthesized by probiotics but they use these compounds in a different way such as the protection against stressful situations, that is, at low pH. Among the neurotransmitters synthesized by probiotics, GABA, serotonin, and dopamine can be highlighted (Ali & Haq, 2010; Wu, Tun, Law, Khafipour, & Shah, 2017).

GABA is a nonprotein amino acid classified as an inhibitory neurotransmitter that mainly works in the central nervous system (CNS) (Sarasa et al., 2019). In most of the situations, *Lactobacillus* spp. uses the GAD pathway to produce GABA. Briefly, this biosynthetic pathway is focused on the decarboxylation of the precursor Glu or its salt monosodium glutamate (MSG). Some probiotics, such as *Aspergillus oryzae*, can use a completely different route where putrescine is used as precursor (Diez-Gutiérrez et al., 2020).

In the same way, other neurotransmitters like serotonin can be produced by probiotics like *L. lactis*, *L. plantarum*, or *Streptococcus thermophilus* (Liang et al., 2019) using Trp as precursor molecule. This amino acid is modified to a 5-hydroxy-tryptophan (5-HTP) by the action of a Trp hydrolase and serotonin is then obtained by bioconversion of 5-HTP catalyzed by an aromatic amino acid decarboxylase (O'Mahony, Clarke, Borre, Dinan, & Cryan, 2015).

Moreover, *A. oryzae* is able to synthesize 3,4-dihydroxyphenyl Ala, better known as L-DOPA, which has positive effects against Parkinson's disease. The enzymatic oxidation of tyrosine (Try) enhances the production of L-DOPA (Ali & Haq, 2010) and some probiotics like *L. lactis* can even use L-DOPA as a precursor of another essential neurotransmitter, dopamine (Vodolazov, Dbar, Oleskin, & Stoyanova, 2018).

2.1.4 Vitamins

Vitamins are micronutrients that play a key role in human health but the inability to produce these compounds forces humans to obtain them exogenously. Several LAB and bifidobacteria can biosynthesize B-group vitamins (Thakur, Sudhir, & Sachinandan, 2016).

Riboflavin, also known as vitamin B_2, presents high relevance in the preservation and restoration of important body structures such as mucous membranes, connecting tissues, neural, or immune system (Ibrahim, Hoda, Kawther, & Sharaf, 2015). Generally, the microbial production of this vitamin involves seven consecutive steps where guanosine triphosphate (GTP) is used as the precursor compound. The process starts with the hydrolysis of the imidazole ring of the GTP and its bioconversion to 5-amino-6-ribitylamino2,4-pyrimidinedione (ARP). Afterward, ARP is transformed by consecutive deamination, reduction of the side chain, phosphorylation, and condensation, which as a result 6,7-dimethyl-8-ribityllumazine (DR). Finally, riboflavin is obtained after dismutation of DR molecule (Bacher, Eberhardt, Fischer, Kis, & Richter, 2000). Thakur et al. (2016) summarized the probiotic bacteria that can release riboflavin naturally. *L. acidophilus*, *Bacillus subtilis*, *Lactobacillus fermentum*, and *L. plantarum* can biosynthesize this vitamin in several dairy products. Riboflavin, also known as vitamin B_2, presents high relevance in the preservation and restoration of important body structures such as mucous membranes, connecting tissues, neural or immune system (Ibrahim et al., 2015). Generally, the microbial production of this vitamin involves seven consecutive steps where GTP is used as the precursor compound. The process starts with the

hydrolysis of the imidazole ring of the GTP and its bioconversion to ARP. Afterward, ARP by deamination, reduction of the side chain, phosphorylation, and condensation consecutive reactions is converted into DR. Finally, riboflavin is obtained after dismutation of DR molecule (Bacher, Eberhardt, Fischer, Kis, & Richter, 2000). Thakur et al. (2016) summarized the probiotic bacteria that can release riboflavin naturally. For instance, *L. acidophilus*, *B. subtilis*, *L. fermentum*, and *L. plantarum* can biosynthesize this vitamin in several dairy products (Thakur et al., 2016).

Folate, vitamin B_{11}, is another remarkable vitamin obtained from probiotic secondary metabolism. This vitamin is interesting due to its participation in the reparation, methylation, and replication of DNA, as well as its implication in illnesses of different etiology such as Alzheimer's, cancer, or coronary diseases, caused by decrease in folate concentration (Ibrahim et al., 2015). The folate biosynthetic pathway is a complex mixture of enzymatic reactions influenced mainly by the stepwise grouping of pteridine, para-aminobenzoic acid (pABA), and Glu. Briefly, the GTP transformation through different steps produces 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) that is linked to pABA by the action of dihydropteroate (DHP) synthase. The fusion of both DHPPP and pABA forms a DHP molecule that is subsequently glutamylated, reduced, and ends up in folate (Rad, Khosroushahi, Khalili, & Jafarzadeh, 2016). Leblanc et al., 2011 reported that *S. thermophilus*, *Bifidobacterium animalis*, or *L. lactis* are able to synthesize high folate concentration.

LAB can produce in less quantity other vitamins from the B-group. Al-Fataftah Abdur Rahman, Herzallah, Mabood, and Alshawabkeh (2013) studied the ability of different LAB to produce vitamin B_{12} (cobalamin) and B_6 (pyridoxine). The results showed that *L. reuteri* obtained from the rumen of goat and camel presented a significant production of these two vitamins (Al-Fataftah et al., 2013). Likewise, Hamzehlou, Abbas, Sedigheh, and Hosseini (2018) evaluated the B-vitamin production of several *Lactobacillus* strains isolated from yogurt reporting that vitamin B_6 and B_9 can be largely synthesized by *Lactobacillus paracasei*. However, vitamin B_3 (niacin) is mainly produced by *L. acidophilus*, and vitamin B_2 by *L. fermentum* (Hamzehlou et al., 2018).

3 Conditions of probiotics to produce postbiotics

Postbiotic production from probiotics depends on precursor concentration, media composition, temperature, pH, or the probiotic incubation time. The effect of each parameter is linked to each probiotic strain and, therefore, these conditions should be specifically adjusted to get the postbiotic target (Diez-Gutiérrez et al., 2020).

3.1 Culture media composition

In general, precursor molecules present a direct relationship between its concentration and the production of postbiotic. For example, Mahara, Lilis, and Hanifah (2019) reported that folate production by most LAB cultures was related to the supplementation of pABA because LAB cannot synthesize de novo this molecule. As well, Shan et al., 2015 indicated that the supplementation of MSG is essential for the synthesis of GABA.

However, some precursor molecules can enhance the synthesis of postbiotics by indirect overstimulation of other biosynthetic routes (Demain, 1998).

Therefore, culture media composition should be balanced between precursor molecule and other additives such as carbon and nitrogen source, cofactors, or polysorbates (Chen et al., 2015a).

Glucose is considered an excellent carbon source because it is easy to metabolize and increases the biomass. In the synthesis of postbiotics, the glucose concentration must be properly set because a high concentration could lead to consuming all this sugar in the growth phase and, in consequence, inhibiting secondary metabolism pathways. Hence, an optimized glucose concentration is required to slow down microorganism growth and focusing on postbiotic production (Ruiz, Chávez, Forero, & García-Huante, 2010). Chen, Wenwen, and Xinmo (2015b) assessed the effect of different carbon sources like glucose, lactose, sucrose, and soluble starch, for GABA synthesis by *L. plantarum*. The results showed higher GABA concentration when glucose was used (Chen et al., 2015a). Zareian, Ebrahimpour, Sabo Mohamed, & Saari, 2013 also considered glucose as the best carbon source for GABA production and they found that the highest amount of GABA was obtained with 6% of glucose.

Nevertheless, glucose is not always the best choice for postbiotic production. For instance, Hernandez-Hernandez et al., 2012 reported that lactulose and galacto-oligosaccharides (GOS) obtained from lactose or lactulose were the best carbon sources for the synthesis of SFCA by some *Lactobacillus* strains.

Moreover, the carbon source supplied can be combined with different nitrogen sources. Wang et al., 2018 evaluated the effects of 36 different nitrogen sources in the biosynthesis of GABA by *Lactobacillus brevis*. The results showed that most nitrogen compounds did not affect GABA production and only some, such as the yeast extracts, increased GABA production (Wang et al., 2018). Ooi et al., 2015 investigated how different nitrogen sources can affect the production of

antimicrobial postbiotics by *L. plantarum.* Interestingly, the combination of glucose and yeast extract increased the concentration of postbiotics, whereas neither glucose and meat extract nor glucose and a cocktail of yeast, peptone and meat extract, had any significant effect on the postbiotic production (Ooi et al., 2015). Ali & Haq, 2010 also studied the effect of different nitrogen sources in the production of L-DOPA by *Aspergillus niger* showing that 6% of glucose combined with 1.5% of peptone and 1% of yeast extract achieved the highest yield.

Another type of additives in the culture medium can have an impact on postbiotic biosynthesis. An example is the emulsifier Tween 80 that increases the membrane fluidization with the incorporation of oleic acid to it and, in consequence, enhances the absorption of nutrients (Foo, Loh, Abdul Mutalib, & Abdul Rahim, 2019). In this sense, Saraniya et al. (2014) showed that Tween 80 is an essential compound for bacteriocin production. Malheiros, Voltaire, Svetoslav, and Bernadette (2015) also highlighted the importance of this emulsifier for bacteriocin production by *Enterococcus faecium* and also reported that other polysorbate emulsifiers such as Tween 20 can enhance the biosynthesis of bacteriocins by *Lactobacillus sakei*.

Regarding other culture medium components, ions and micronutrients added in low amount could improve the postbiotic synthesis. For example, vitamin B_{12} biosynthesis is specifically influenced by cobalt and several minor nutrients such as tripotassium phosphate, manganese chloride, or sodium phosphate (Kośmider, Bialas, Kubiak, Drozdzynska, & Czaczyk, 2012). Lim et al., 2018 studied the effect of different chemical reagents and coenzymes on the expression of GAD enzyme used in GABA synthesis. Among coenzymes, pyridoxal-5-phosphate (PLP), pyridoxal hydrochloride, and pyridoxine were added to determine which of these compounds have the highest impact on GABA yield. The highest amount of GABA was obtained using PLP followed by pyridoxine and pyridoxal hydrochloride. Focusing on chemical reagents, better results were obtained using calcium chloride, ammonium sulfate, or manganese chloride, among others (Lim et al., 2018).

3.2 Cultivation parameters

Temperature, incubation time, and pH of the culture medium can play a key role in the postbiotic synthesis and, therefore, the optimization of these parameters in combination with other conditions can enhance the postbiotic production (Zhang, Zeng, Tan, Tang, & Xiang, 2017). Leblanc et al., 2011 reported high concentration of folate obtained at 30°C during 4 days of incubation. Additionally, the intracellular pH could affect folate production depending on the probiotic strain. For example, acidic environments could help *S. thermophilus* to produce more amount of folate but this condition was not relevant for *L. lactis* (Leblanc et al., 2011). Min, Kyungmoon, Don, and Young Je (2015) found that the synthesis of L-DOPA could be maximized at pH 8 and 40°C allowing *Bacillus* sp. *JPJ* to achieve a 99.4% bioconversion of Tyr into L-DOPA.

Miao et al., 2015 performed an optimization of the production of an antimicrobial postbiotic by *L. paracasei.* The best results were shown after 24 h of incubation, at 30°C and an initial pH of 7 (Miao et al., 2015). Zareian et al., 2012 reported high concentration of Glu after 96 h of incubation, at 30°C, and 4.5 as initial pH. Likewise, Tajabadi et al., 2015 determined that *L. plantarum* produced more GABA after 60 h of incubation, at 36°C, and an initial pH around 5.

4 Human health benefits of probiotics and postbiotics

Microbiota is the term used to designate microorganisms that live in a specific environment, called itself a microbiome. These microorganisms can be commensal, symbiotic, and pathogenic bacteria, fungi, and viruses. In the case of microorganisms that grow in the intestine, they are called the gut microbiota. Recent studies have investigated that the microbiome is capable of modulating behavior by connecting the neuroendocrine and immune systems (Sylvia et al., 2018). In addition, the existence of the so-called gut–brain axis has been demonstrated, in which the microbiota of the digestive tract and the CNS are bidirectionally connected. In this regard, this connection is believed to occur through three pathways: the vagus nerve, the systemic pathway (by releasing hormones, metabolites, and neurotransmitters), and the immune system (by the action of cytokines), as it can see in Fig. 17.2 (Molska & Reguła, 2019; Gómez-Eguílaz, José, Laura, & Blanco, 2019).

In a healthy gut microbiota, there are about 500 different species, bacteria, and fungi that cause disease along with beneficial bacteria. If the latter bacteria prevail, the intestinal physiology is normal, and the overall body status is healthy. Among the causes that may cause the disappearance of beneficial bacteria from the intestinal microbiota are stress, infections, antibiotic treatments, inadequate diets, etc.

Dysbiosis is the imbalance between gut microorganisms that generates a deteriorated microbiota. Pathogenic bacteria, viruses, yeasts, and fungi grow out of control, often leading to a rise in allergies and autoimmune conditions (Fig. 17.2) (Lyte, 2014).

Previous studies reported that intestinal microbiota is responsible for producing neurotransmitters such as dopamine, serotonin, and norepinephrine (Lyte, 2011), and that these compounds can directly reach the brain through the vagus nerve

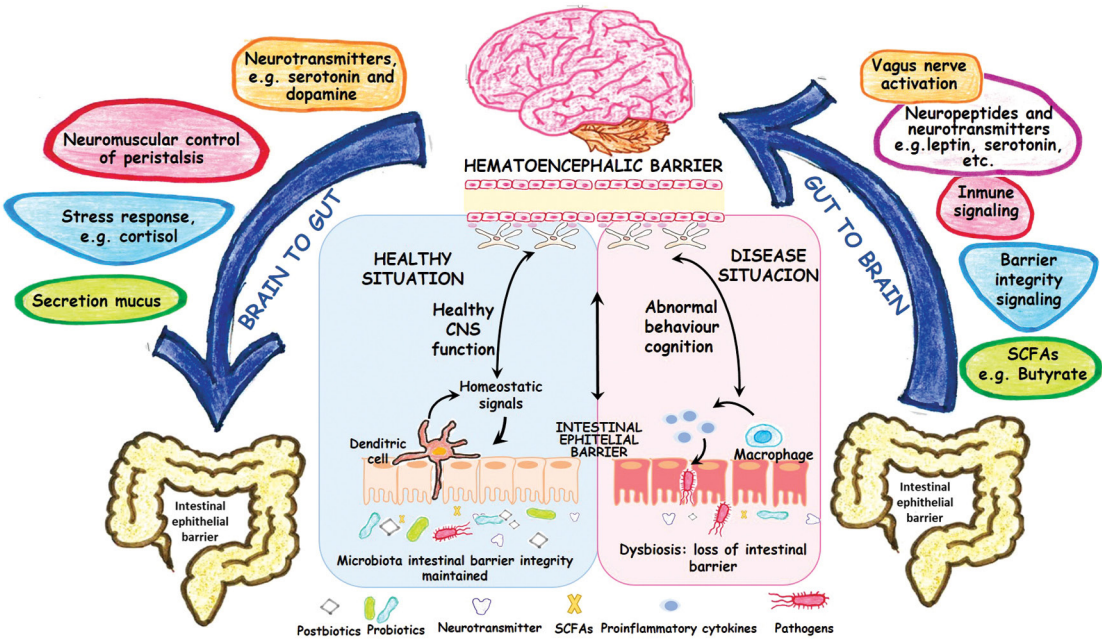

FIGURE 17.2 Gut–brain connection. Scheme depicting the gut–brain axis connections that can be activated or modulated by stress and diseases.

(Perez-Burgos et al., 2013; Borovikova et al., 2000). Also, high levels of neurotransmitter precursors such as Trp have been linked to gut microbiota (Desbonnet, Garrett, Clarke, Bienenstock, & Dinan, 2008), as well as the activation of neuroreceptors associated with appetite control, pain sensation, mood, and memory (Muccioli et al., 2010). In 2017 Anderson, Cryan, & Dinan, 2017 created the term "psychobiotics" to explain the benefit of consuming adequate amount of live probiotics for a good psychological health. Since then, this term has been expanded to include both probiotic microorganisms and postbiotic compounds.

Currently, there are a large number of scientific studies that demonstrate the relationship between probiotics and postbiotics and their effect on human physiology and health, as it can see in Table 17.3.

Although many unknown aspects still need to be clarified, the gut–brain axis is postulated as responsible for numerous neurological disorders of great health impact, such as Alzheimer's disease, Parkinson's disease, or multiple sclerosis, as well as other diseases such as cancer, diabetes, asthma, and intestinal bowel disease.

Currently, scientific studies, such as cell cultures, in animal models, human clinical trials, are underway trying to assess the impact of probiotics and postbiotics on some of these diseases.

5 Application of probiotics and postbiotics for healthy food development

Once the beneficial effects of the consumption of probiotics and postbiotics have been recognized, it is necessary to define the way in which they will be consumed. In the market, there are different examples of the commercial strategies that are chosen both in the pharmaceutical industry (in the parapharmacy product line) and in the food industry: nutraceutical formulations in the form of capsules, tablets, dispersible powders, etc. or as ingredients that are incorporated in food and beverages.

The introduction of probiotics in food formulation has found no barriers for consumers, since food fermentation is an ancient strategy to extend the shelf life of food and also involves a flavor and texture modification of the raw material creating and interesting food and cultural heritage: cheese (historically from Middle East), miso (Japan), wine (Zagros mountains), vinegar (Roman empire), chili sauce (Mexico), kombucha tea (China), sauerkraut (Germany), kimchi (Korea), fermented fish (Asia), garri (Nigeria), etc.

In addition, nowadays, fermented foods are related to healthy diets and modern cuisine. According to Kerry Health and Nutrition Institute (2020), the fermented food product market is projected to reach $689.34 billion by 2023, and the CAGR (Compound Annual Growth Rate) is expected to be between 4.3 and 7%, being Europe and United States the largest markets and Asia-pacific the fastest growing one. In fact, there is a positive perspective on fermented foods and beverages from a large part of the consumers with food taste and health aspects important purchase incentives.

TABLE 17.3 Probiotics and postbiotics with beneficial human health effects.

Probiotic/Postbiotic		Disease	Human health effect	References
PROBIOTICS				
Probiotics	*Lactobacillus acidophilus*	Intestinal bowel disease, colorectal cancer Crohn's disease, ulcerative colitis L.	Enhances immune response ↑*Lactobacilli*, *Bifidobacteria* ↓ *Staphylococcus aureus*	Chen et al. (2015b), Chen, Zou, & Lian, (2013), Khazaie et al., (2012), Park et al., (2018)
	Lactobacillus casei BL23	Colorectal cancer	Expand gut Treg cells Enhances immune response	Jacouton, Chain, Sokol, Langella, & Bermúdez-Humarán, (2017), Lozano-Ojalvo, Leblanc, & Bermúdez-Humarán, (2016)
	Lactobacillus fermentum FTDC 812	Hypercholesterolemia	↑*Lactobacillus*	Lye et al., (2017)
	Lactobacillus johnsonii	Acute live injury	↑IL-22, *Lactobacillus*	Nakamoto et al., (2017)
	Lactobacillus plantarum CCFM10, RS15-3	Oxidative stress	↑*Bacteroidetes*, *Firmicutes*	Zhao et al., (2018)
	L. acidophilus Lactobacillus rhamnosus, *Bifidobacterium bifidum*	Type 2 diabetes	↑*Firmicutes*, *Actinobacteria* ↓*Bacteroidetes*	Bagarolli et al., (2017)
	Bifidobacterium breve IPLA20004 E.	Inflammatory	Enhances immune response	Sánchez et al., (2015)
POSTBIOTICS				
SCFA	Short-chain fatty acids	Multiple sclerosis	Promote T-cell differentiation toward regulatory subtypes (Treg cells)	Zeng, Gong, Liu, & Chen, (2019)
	Short-chain fatty acids	Type 2 diabetes	Enhance glucose homeostasis and insulin effectiveness	Mandaliya et al. (2019)
	Butyrate	Autoimmune diseases	Facilitating neuronal plasticity and long-term memory formation Restoring cognitive function	Haghikia et al., (2015)
	Propionate	Inflammatory diseases	Protective effects against lipopolysaccharides (LPS), induced blood–brain barrier disruption, and oxidative stress	Chen et al., (2017), Hoyles et al., (2018)
Protein/peptides/amino acids	Tryptophan	Intestinal bowel disease	5-Hydroxytryptamine, kynurenine, and aryl hydrocarbon receptor pathways	Agus, Planchais, & Sokol, (2018)

(*Continued*)

TABLE 17.3 Probiotics and postbiotics with beneficial human health effects. (*Cont.*)

Probiotic/Postbiotic		Disease	Human health effect	References
Neurotransmitters	GABA	Heart attack and stroke	Hypotensive effect	Abd El-Fattah, Sakr, El-Dieb, & Elkashef, (2018), Cáceres, (2017)
	GABA	Huntington's disease Alzheimer's disease	Inhibits neurotransmission	Fuhrer et al., (2017), Hsu, Chang, & Chern, (2018), Mele, Rui, and Duarte (2019)
	GABA	Anxiety and depression	Relaxant and antidepressant effect	Boonstra et al., (2015), Bravo et al., (2011), Soussan et al. (2016)
	GABA	Epilepsy	Reduce seizure severity	Bagheri, Heydari, Alinaghipour, & Salami, (2019)
	GABA	Diabetes type 1	α-Cells: GABA induces membrane hyperpolarization and inhibits glucagon secretion. β-Cells: GABA induces membrane depolarization and enhances insulin secretion	Tian, Lu, Zhang, & Chau, (2014), Qinghua, Gerald, and Yun (2015)
	GABA	Cancer	Delays and/or inhibits cancer cell proliferation Stimulatory action on cancer cell apoptosis Potent tumor suppressor	Brzozowska, (2017), Song et al., (2016), Wang et al. (2016b)
	GABA	Asthma	Control in asthma Enhances immunity	Yocum et al., (2017), Forkuo et al., (2017)
	L-DOPA	Parkinson's disease	Control dopamine deficiency	Min et al. (2015)
	Dopamine	Neural diseases	Regulation neurochemical pathways	Lyte (2018)
	Acetylcholine	Alzheimer's disease	Control acetylcholine deficiency and cholinergic receptors alteration	Nimgampalle et al. (2017)
	Acetylcholine and serotonin	Cancer	Regulation neural signaling and inflammatory response	Gayathri (2016)

One of the health benefits perceived by consumers is the transformation of raw food components to make healthier foods by postbiotic compound production. The ingestion of FODMAP (Fermentable Oligo-, Di-, Monosaccharides And Polyols) is related to gastrointestinal disturbances and a low FODMAP diet could be especially targeted at people with functional bowel disorders such as irritable bowel syndrome (Ramírez, Tejero Mas, Gato Núñez, Rivera Jiménez, & Román Vargas, 2018). FODMAP includes components present in different foods and beverages, such as milk or plant-based ones: fructans and GOS, lactose, fructose, and polyalcohols. Food fermentation is an alternative for removing FODMAP in foods (Nyyssölä, Simo, Emilia, & Poutanen, 2020). Thus *Saccharomyces cerevisiae* activity contributes to reduce the FODMAP content in dough during fermentation. Fermentation is also a useful strategy to reduce the presence of GOS in legumes, such as soybean fermented products (tofu, tempeh, miso, soy sauce, etc.) or the lactose reduction in fermented dairy products such as yogurt, fermented milk, or cheese by LAB activity.

On the other hand, consumers also appreciate fermented foods as healthy due to the ability of microorganisms to synthesize bioactive compounds. For example, *Acetobacter* and *Gluconobacter* oxidize ethanol from alcoholic beverages to produce vinegar and the acetic acid produced has been related to the glucose metabolism, showing a beneficial effect on the glycemic profile (Santos, Moraes, da Silva, & Prestes, 2019).

The healthier perception of fermented foods is driving an important evolution in the food and beverage marketplace, particularly for dairy products. However, plant products are increasing rapidly in the global food market and according to Allied Market Research (2019), vegan food market size is expected to reach $31.4 billion by 2026. Nowadays, the food and beverage industry is working to standardize traditional and new fermented products in order to produce healthy fermented foods according to consumer demands under safety and quality requirements in their target markets (Adewumi, 2019; IsamMohamed Ahmed & FahadAl-Juhaimi, 2019). But also, scientific and technological advances are being to improve fermented food products to the market.

However, to ensure the functionality of new products, it is necessary to ensure the concentration of probiotics and postbiotics in the selected dosage form. This implies that the active compounds must not interact with other components of the product matrix throughout its useful life and it must remain unchanged after ingestion.

One remarkable technology applied to improve probiotics and postbiotics functionality is the encapsulation. Microencapsulation of functional components is a process of entrapping components within one or more classes of shell materials to fabricate a capsule, typically a few microns in diameter referred to microcapsules. The microencapsulation is used to enhance nutritional value, mask off-flavor, facilitate storage, and extend shelf life without adverse influence on the food physical, chemical, or functional properties (Ye, Nicolas, & Selomulya, 2018). Nanoencapsulation is generally defined as the design, production, and application of structures, devices, and systems, through control of the shape and size of the material between 1 and 100 nm in order to achieve the delivery of poorly bioactive compounds into functional food ingredients (Bazana, Codevilla, & de Menezes, 2019).

Currently, there is an extensive number of encapsulation technologies that can serve to encapsulate probiotics to optimize their viability both in the fermentation process and in the final product for controlled release in the intestine (Chávarri, Izaskun, & Maria, 2012). Maintenance and viability of starter culture in fermented foods is still an immense challenge for the food industry. Starter culture encapsulation provides protection to the cells increasing the viability of the delivered amount (Kavitake, Sujatha, Palanisamy, & Shetty, 2018) or the delivery of metabolites produces by the encapsulated cell (Barbosa, Todorov, Jurkiewicz, & Bernadette, 2015; Lindner et al., 1998), but also opens possibilities to optimize selected probiotic starters for other applications (Plessas et al., 2007).

Furthermore, these micro- and nanoencapsulation technologies can be used also to improve the stability of postbiotic compounds and their bioavailability. Microencapsulated postbiotics could avoid degradative reactions during storage, such as oxidation (Rasti, Arezoo, & Selamat, 2017); on the other hand, the application of enteric coating materials provides gastric resistance to minimize the degradation of postbiotic compounds in the gastric acid medium (Puccetti, Giovagnoli, Zelante, Romani, & Ricci, 2018); and encapsulation is also used to control the delivery of postbiotic compounds in a specific targeted point of the human body (Fontes et al., 2018). All encapsulating materials must be food grade or pharmaceutical grade to develop a food ingredient or nutritional complement dosage form, and they are normally selected between polysaccharides, proteins, lipids, waxes, etc. These materials are appropriately selected regarding the encapsulation technology and the desired release mechanism (Table 17.4).

Moreover, encapsulation technologies are a useful tool to adjust the dosage of functional ingredients when a probiotic culture must be dosed with a specified amount of prebiotic ingredient (Marañon García, San Vicente Laurent, Hidalgo Lemus, & Chavarri, 2016) or metabolite (Eratte et al., 2015), as well as to accurately dose a defined proportion of several cultures (Divya et al., 2015).

As mentioned previously, beyond their relevance in the scientific field, postbiotics and probiotics are important for food industry. According to the report published by Grand View Research in April 2019, the global functional food market size was

TABLE 17.4 Examples of some encapsulation strategies for postbiotics.

Postbiotic	Material	Encapsulation technology	References
SCFA	Cellulose nanocrystals, sunflower oil	Pickering emulsions	Du Le, Simon, Loveday, and Sarkar (2020)
	Lecithin, sunflower oil	Nanoliposomes	Ghorbanzade, Seid Mahdi, Sahar, and Hadavi (2017)
	PEG, cholesterol, phosphatidylcholine	Liposomes	Rasti et al. (2017)
	Whey protein isolate, Arabic gum	Complex coacervation	Eratte et al., (2015)
Amino acids; peptides	Chitosan	Gelation	Danish, Vozza, Byrne, Frias, & Ryan, (2017)
	Niosomes; Liposomes	Nanovesicles	Rezvani et al., (2019)
	Maltodextrin	Spray-drying	Akbarbaglu et al., (2019)
	Whey protein	Gelation	O'Neill, Egan, Jacquier, O´Sullivan, & O´Riordan, (2014)
Neurotransmitters	PEG, cholesterol, phosphatidylcholine	Liposomes	Fontes et al., (2018)
	Chitosan	Ionic gelation method	Shilpa, Mary, Malat, and Paulose (2013)
Vitamins	Whey protein, starch	Gelation	Liu et al., (2020)
	Phospholipids	Nanoliposomes	Hamadou, Wen-Can, Changhu, and Xiangzhao (2020)
	Ovalbumin, pectin	Complex coacervation	Xiang et al., (2020)
	Triglycerides, lecithin, surfactants, etc.	Nanoemulsion	Maurya and Aggarwal (2019)

estimated at $161.49 billion in 2018, and it is projected to reach $275.77 billion in 2025 (FFMW, 2019). Regarding functional ingredients, the probiotic market size was estimated at $2.09 billion in 2018 with a projected CAGR of 7.9% for the period 2019–25 (Grand View Research, 2020). Postbiotics are not recognized as functional ingredients but they are relevant considering that, for example, SCFAs or vitamins are some of the most relevant ingredients to determine food market perspectives.

6 Conclusions

Probiotics are currently in the spotlight of the food field due to the wide variety of potential beneficial effects particularly useful in maintaining human welfare. Likewise, the research and production of postbiotics are evolving in such a way that these beneficial metabolites are gaining importance because their broad spectrum of health action combined with the effectiveness of probiotics goes one step further in the development of new functional foods.

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ANNEX III: PATENT INFORMATION

Europäisches Patentamt
European Patent Office
Office européen des brevets

Request for grant of a European patent

For official use only		
1 Application number:	MKEY	
2 Date of receipt (Rule 35(2) EPC):	DREC	
3 Date of receipt at EPO (Rule 35(4) EPC):	RENA	
4 Date of filing:		

5 Grant of European patent, and examination of the application under Article 94, are hereby requested. ☒

Request for examination in an admissible non-EPO language: Se solicita el examen de la solicitud según el artículo 94.

5.1 The applicant waives his right to be asked whether he wishes to proceed further with the application (Rule 70(2)) ☐

Procedural language: en

Filing Language: en

6 Applicant's or representative's reference: P5663EP00

Filing Office: ES

Applicant 1

7-1 Name: FUNDACION TECNALIA RESEARCH & INNOVATION

8-1 Address: Parque Científico y Tecnológico de Gipuzkoa
Paseo Miramón, 2
20009 SAN SEBASTIÁN
Spain

10-1 State of residence or of principal place of business: Spain

14.1 The/Each applicant hereby declares that he is an entity or a natural person under Rule 6(4) EPC. ☒

Representative 1

15-1	Name:	ZBM Patents - Zea, Barlocci & Markvardsen
	Association No.:	371
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Inventor(s)

23 Designation of inventor attached ☒

24 Title of invention

Title of invention: MICROCAPSULES CONTAINING GAMMA-AMINOBUTYRIC ACID

25 Declaration of priority (Rule 52) and search results under Rule 141(1)

A declaration of priority is hereby made for the following applications

25.2 Re-establishment of rights

Re-establishment of rights under Article 122 EPC in respect of the priority period is herewith requested for the following priority/priorities

25.3 The EPO is requested to retrieve a certified copy of the following previous application(s) (priority document(s)) via the WIPO Digital Access Service (DAS) using the indicated access code(s):

Request	Application number:	Access Code

25.4 This application is a complete translation of the previous application ☐

25.5 It is not intended to file a (further) declaration of priority ☒

26 Reference to a previously filed application

27 Divisional application ☐

28 Article 61(1)(b) application ☐

29 Claims

Number of claims: 15

29.1 ☒ as attached

29.2 ☐ as in the previously filed application (see Section 26.2)

29.3 ☐ The claims will be filed later

30 Figures

It is proposed that the abstract be published together with figure No. [ ]

31 Designation of contracting states

All the contracting states party to the EPC at the time of filing of the European patent application are deemed to be designated (see Article 79(1)).

32 Different applicants for different contracting states

33 Extension/Validation

This application is deemed to be a request to extend the effects of the European patent application and the European patent granted in respect of it to all non-contracting states to the EPC with which extension or validation agreements are in force on the date on which the application is filed. However, the request is deemed withdrawn if the extension fee or the validation fee, whichever is applicable, is not paid within the prescribed time limit.

33.1 It is intended to pay the extension fee(s) for the following state(s): [ ]

33.2 It is intended to pay the validation fee(s) for the following state(s): [ ]

34 Biological material

38 Nucleotide and amino acid sequences

38.1 ☐ The description contains a sequence listing.

38.2a ☐ The sequence listing is attached in computer-readable format in accordance with WIPO Standard ST.25 (Rule 30(1)).

38.2b ☐ The sequence listing is attached in PDF format

Further indications

39 Additional copies of the documents cited in the European search report are requested

Number of additional sets of copies: [ ]

40 ☐ Refund of the search fee under Article 9(2) of the Rules relating to Fees is requested

Application number or publication number of earlier search report: [ ]

42 Payment

Method of payment: Debit from deposit account

The European Patent Office is hereby authorised, to debit from the deposit account with the EPO any fees and costs indicated on the fees section below.

Currency:	EUR
Deposit account number:	28120033
Account holder:	Anna Barlocci

43 Refunds

Any refunds should be made to EPO deposit account:	28120033
Account holder:	Anna Barlocci

Fees	Factor applied	Fee schedule	Amount to be paid
001 Filing fee - EP direct - online	1	125.00	125.00
002 Fee for a European search - Applications filed on/after 01.07.2005	1	1 350.00	1 350.00
015 Claims fee - For the 16th to the 50th claim	0	245.00	0.00
015e Claims fee - For the 51st and each subsequent claim	0	610.00	0.00
501 Additional filing fee for the 36th and each subsequent page	0	16.00	0.00
Total:		**EUR**	**1 475.00**

44-A Forms		Details:	System file name:
A-1	Request		as ep-request.pdf
A-2	1. Designation of inventor	1. Inventor	as f1002-1.pdf

44-B Technical documents		Original file name:	System file name:
B-1	Specification	P5663EP00_filing.pdf Description; 15 claims; 2 figure(s); abstract	SPECEPO-1.pdf
B-3	Pre-conversion archive	P5663EP00_filing_pre.zip	OLF-ARCHIVE-1.zip
B-4	Translation of description, claims, abstract and drawings in Spanish	P5663EP00_filing_resumen_ES.pdf	SPECTRANONEP.pdf

44-C Other documents	Original file name:	System file name:

45	General authorisation:	

46 Signature(s)

Place:	**Barcelona**
Date:	**22 June 2021**
Signed by:	**Mireia Cama 33265**
Association:	**ZBM Patents - Zea, Barlocci & Markvardsen**
Representative name:	**Mireia Cama**
Capacity:	**(Representative)**

Form 1002 - 1: Public inventor(s)

Designation of inventor

User reference: P5663EP00
Application No:

Public

	Inventor	
	Name:	CHÁVARRI HUEDA María Blanca
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	Inventor	
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	The applicant has acquired the right to the European patent:	As employer
	Inventor	
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	The applicant has acquired the right to the European patent:	As employer

Signature(s)

Place:	**Barcelona**
Date:	**22 June 2021**
Signed by:	**Mireia Cama 33265**
Association:	**ZBM Patents - Zea, Barlocci & Markvardsen**
Representative name:	**Mireia Cama**
Capacity:	**(Representative)**

Form 1002 - 1: Public inventor(s)

Designation of inventor

User reference: P5663EP00
Application No:

Public

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	The applicant has acquired the right to the European patent:	As employer
	Inventor	
	Name:	DIEZ GUTIÉRREZ Lucía Camino
	Address:	01510 MIÑANO Spain
	The applicant has acquired the right to the European patent:	As employer

Signature(s)

Place:	**Barcelona**
Date:	**22 June 2021**
Signed by:	**Mireia Cama 33265**
Association:	**ZBM Patents - Zea, Barlocci & Markvardsen**
Representative name:	**Mireia Cama**
Capacity:	**(Representative)**

MINISTERIO
DE INDUSTRIA, ENERGÍA
Y TURISMO

Oficina Española
de Patentes y Marcas

Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

Submission number	300414218	
Application number	EP21382550.8	
File No. to be used for priority declarations	EP21382550	
Date of receipt	22 June 2021	
Your reference	P5663EP00	
Applicant	FUNDACION TECNALIA RESEARCH & INNOVATION	
Country	ES	
Title	MICROCAPSULES CONTAINING GAMMA-AMINOBUTYRIC ACID	
Documents submitted	package-data.xml	ep-request.xml
	application-body.xml	ep-request.pdf (5 p.)
	OLF-ARCHIVE-1.zip\P5663EP00_filing_pre.zip	SPECTRANONEP.pdf\P5663EP00_filing_resumen_ES.pdf (1 p.)
	SPECEPO-1.pdf\P5663EP00_filing.pdf (34 p.)	f1002-1.pdf (1 p.)
Submitted by	CN=Mireia Cama 33265	
Method of submission	Online	
Date and time receipt generated	22 June 2021, 15:35:46 (CEST)	
Official Digest of Submission	44:10:82:EE:15:76:CA:C2:95:20:A0:B8:F0:2D:1B:07:B5:66:05:8C	

/Madrid, Oficina Receptora/


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Universidad
del País Vasco
Euskal Herriko
Unibertsitatea