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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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