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A novel antidiabetic lactofermented beverage from agro-industrial waste (broccoli leaves): Process optimisation, phytochemical characterisation, and shelf-life through thermal treatment and high hydrostatic pressure

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ABSTRACT

Fermentation is the main process in several foods claimed to be health-promoting products due to their content in microorganisms and bioactive compounds. Therefore, in this study, the fermentation process was used to revalorise broccoli waste in the development and optimisation of a new fermented beverage by *Lactiplantibacillus plantarum* following a fermentation-assisted extraction strategy. The beverage was also subjected to several processing treatments and stored at 5 °C for 49 days. LC-MS/MS was used to characterise and quantify the individual (poly)phenolic and isothiocyanate contents. Optimised fermentation was carried out using a Box-Behnken design and the process was set to 4 days, 0.1 g of leaves per mL and 27.77 °C. Pasteurisation resulted in a higher (poly)phenolic content but caused a fall in LAB population to 3.5 log CFU/mL. In the control beverage, the (poly)phenol content decreased with increasing storage time and the LAB population was maintained at 6.5 log CFU/mL. In the beverage treated with high hydrostatic pressure (HHP 200 MPa, 10 min) the LAB population also remained like the control, maintained the stability of the phytochemical with the highest content of sulforaphane (4.38–8.82 mg/L), and a high stability of its antidiabetic potential during refrigeration. In contrast, the beverage subjected to HHP 400 MPa for 1 min had reduced LAB population (2 log CFU/mL), and obtained the lowest (poly)phenolic content, and α-amylase inhibition. In conclusion, the results suggest that HHP 200 MPa treatment was the most suitable method to achieve stability of LAB population and phytochemicals throughout the shelf-life.

1. Introduction

Fermentation is an ancient method for food preservation which has been used for a wide range of foodstuffs such as fish, dairy products, legumes, cereal, fruits, and vegetables (Wilburn & Ryan, 2017). During the fermentation process, microorganisms such as yeasts, moulds, or lactic acid bacteria (LAB) transform the flavour profile and enhance sensory properties, including texture, colour, and aroma, whilst nutrient digestibility and biofortification are improved (Salas-Millán et al., 2023; Tamang, 2012, pp. 44–55). LAB play a crucial role as essential microorganisms utilised for probiotic applications and are commonly used in the food industry as either starter or autochthonous cultures. Species such as *Lactobacillus acidophilus*, *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, and *Lacticaseibacillus rhamnosus* are frequently present in plant-based fermented foods (Lahtinen et al., 2011). LAB are responsible for the production of various fermented foods, such as yoghurt, cheese, sausages, and fermented vegetables like cabbage, cucumber, and pepper. Notable examples include juices fortified with probiotics derived from fruits and vegetables. There is growing consumer demand for food and drink products that promote wellbeing, leading to an increase in the production of functional foods (Ayed et al., 2020).

Fermentation processes involve a series of transformations that can enhance the stability and shelf-life of the food through the production of organic acids (pH reduction), alcohol, antimicrobial peptides, and other compounds. However, food manufacturers must ensure microbial stability throughout the shelf-life of their products to provide safe and high-quality food. Thermal treatment is a widely used method to improve

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microbial stability in food but can lead to the loss of heat-sensitive nutrients, vitamins, and bioactive compounds; sensorial quality deterioration; and also compromise the perception of freshness (Venzke Klug et al., 2020). However, high hydrostatic pressure (HHP) is an emerging non-thermal food preservation technique that uses elevated pressure (100–800 MPa) for a short time (3–15 min) to achieve microbial inactivation and thereby extend the shelf-life of foods (Morales-de la Peña et al., 2023). HHP enables the preservation of heat-sensitive compounds, minimising the impact on sensory and nutritional attributes (Salar et al., 2023). Additionally, by adjusting parameters such as pressure and time, it is possible to maintain the sensory features and shelf-life of fermented products, whilst also preserving optimal LAB population levels (Pega et al., 2018).

On the other hand, discarded material from the fruit and vegetable industry represents a significant volume of bio-residues that contain valuable functional compounds such as fibre, protein, peptides, vitamins, polyphenols, and glucosinolates (in Brassica species) (Domínguez-Perles et al., 2010). These compounds offer notable health benefits and have the potential to reduce the incidence and progression of chronic diseases such as diabetes, obesity, cardiovascular diseases, and intestinal disorders (Gil-Martín et al., 2022).

To address this issue, strategic actions have been put in place to minimise the waste of valuable resources. These measures focus on approaches that include food waste reduction, the adoption of zero waste practices, and the revalorisation of by-products (Bos-Brouwers et al., 2023). Such strategies not only promote a circular economy but also support initiatives to achieve a low carbon footprint in the industry. Additionally, by-product valorisation contributes to overall efficiency improvements within the food sector (Salas-Millán et al., 2022), and therefore contributes to avoiding the waste of critical resources such as water, land, and energy.

In this paper, we will work on broccoli byproducts since the annual worldwide production of cauliflower and broccoli exceeds 25 million tonnes (FAOSTAT, 2020). Broccoli (*Brassica oleracea* var. *italica*) is very rich in functional compounds including phenols, vitamin C and glucosinolates (Artés-Hernández et al., 2023). However, only 15% of the total biomass in broccoli is useable, whilst the roots, stalk, and leaves are wasted, under-using about 17, 21, and 47%, respectively (Liu et al., 2018) with the consequent waste of limited natural resources. Some authors have used broccoli waste in the production of sauces, bakery products and pickles, with the main objective being the valorisation of the waste for the development of new products (Castillejo et al., 2021; Drabińska et al., 2018; Salas-Millán et al., 2022), and broccoli leaves to enrich green tea beverages (Dominguez-Perles et al., 2011). Moreover, Sun et al. (2023) revalorised broccoli stems and leaves through fermentation, mixing them with other cereals to obtain animal feed.

For all of the above reasons, the aim of the present research was to revalue broccoli leaves using the fermentation process as the basis of a means of assisted extraction to develop a lactofermented beverage with a high LAB population and total phenolic compounds and antioxidant capacity potential. Furthermore, in the second part, the optimised beverage was subjected to thermal and HHP treatments to determine the LAB population, stability of its phytochemicals, and antidiabetic potential during shelf-life (49 days at 5 °C).

2. Materials and methods

2.1. Main equipment and bacterial strains for fermentation-assisted extraction

The experiments were conducted using a Bionet F0–2CC bench-top bioreactor (Bionet, Fuente Álamo, Spain) with a nominal volume of 2.2 L and a total volume of 3.4 L. The bioreactor has dimensions of 459 x 220 × 212 mm, with an inner height and diameter of 249 mm and 135 mm, respectively. The H/D ratio is 1.84, with a turbine diameter of 54 mm. The pH and dissolved oxygen levels were monitored online using ROSITA software. The fermented liquid was extracted aseptically using an autoclaved rubber tube and a Percom N-M II peristaltic pump (J.P. Selecta, Abrera, Spain) in a laminar flow hood.

2.2. Bacterial strains and cultures

The probiotic strain *Lactiplantibacillus plantarum* (CECT 749), was used to ferment agro-industrial broccoli waste (leaves). To prepare the inoculum, 1 mL of thawed stock culture was inoculated into 50 mL of sterile MRS broth and incubated under anaerobic conditions at 37 °C for 12–14 h until it reached the stationary phase (the cell population reached 9 log CFU/mL). Before adding the inoculum (6 log CFU/mL) to the bioreactor, *L. plantarum* was washed in Ringer's solution.

2.3. Fermentation–assisted extraction of broccoli leaves

The broccoli leaves (*Brassica oleracea* L. *var Italica, cv. Parthenon*) used in this study were obtained from the Levante Sur Coop. in south-eastern Spain (La Palma, Cartagena). The leaves were washed with tap water, cut into 2 cm squares, and air dried in a salad spinner. The leaves (solid) were placed in an autoclaved bioreactor jar with mineral water (solvent) and inoculated with *L. plantarum* (6 log CFU/mL). The fermentation process was carried out at specific temperatures, solid/solvent ratios, and times, as detailed in section 2.4.

2.4. Box-Behnken design experiment (BBD)

The Box-Behnken design (BBD) was used to develop and optimise the fermentation–assisted extraction method. The study focused on three independent factors (Table 1), which are considered to be the most significant in the fermentation process: X_1, the fermentation time (days): 2, 3, 4; X_2, the solid-solvent ratio (g of broccoli leaves per mL of water): 0.05, 0.075, 0.1; and X_3, the temperature (°C): 25, 31, 37.

The study included the effects of these factors on the multi-response variables, including total phenolic content (TPC), Trolox equivalent antioxidant capacity (TEAC), and LAB count. The experimental design incorporated three levels per factor (Table 1): a lower level (−1), an intermediate level (0), and a higher level (1). The design included a set of points located at the centre of each edge in a multidimensional cube and replicates at the centre point (n = 3). The resulting response data from TPC, TEAC, and LAB counts were used in the mathematical model to fit in a second-order polynomial function:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \varepsilon$$

Where Y represents the different responses (Y_{TPC}, Y_{TEAC}, and Y_{LAB}), β_0 is the model constant, β_i the coefficient for each main effect, β_{ij} the coefficient corresponding to the interactions between factor i and factor j, β_{ii} the coefficient of the quadratic factors that represent the curvature of the surface, and X each factor studied (time, solid-solvent ratio, and temperature). The model also accounts for random error ε.

The significant variables for TPC, TEAC, and LAB were obtained using Standardised Pareto charts (Fig. 1). These charts display the effects in decreasing order of significance, with a line to determine which effects are statistically significant. The length of each bar of the Pareto chart is proportional to the value of a t-statistic and is calculated by dividing the corresponding estimated effect by its standard error. The chart's vertical line highlights the factors or combinations of factors that significantly affect each compound's response. The vertical line's value is calculated using the student's t-test parameter for $p = 0.05$. Any bars beyond this critical value are statistically significant at the selected confidence level of 95%.

Table 1
Box-Behnken design for the three independent variables (TPC, TEAC and LAB), with their constant and coefficients (β).

Factors	Unit	-1	0	+1
Time (X_1)	Days	2	3	4
Solid-solvent ratio (X_2)	(w/v)	0.05	0.075	0.1
Temperature (X_3)	°C	25	31	37

Coefficient	TPC	TEAC	LAB
β_0	332.56	0.315191	-2.12331
β_1	-159.156	-0.168473	-0.944779
β_2	-131.085	-2.74665	62.2148
β_3	4.2129	0.0279485	0.712089
β_{11}	11.8177	0.00901918	0.180346
β_{12}	660.972	1.50018	-3.23752
β_{13}	1.9022	0.00128359	0.00187551
β_{22}	15702.8	36.5923	-368.811
β_{23}	-51.2588	-0.074792	0.161031
β_{33}	-0.169004	-0.000652793	-0.0128191
R^2	0.977842	0.97486	0.952242
R^2-adj	0.937957	0.929607	0.866278

Codification of independent ranged factors: X_1, time in days; X_2, solid-solvent ratio as g of broccoli leaves per mL of water (w/v); X_3, temperature in °C. TPC: Total phenolic content; TEAC: Trolox equivalent antioxidant capacity; LAB: Lactic acid bacteria count.

2.5. Processing treatments and storage conditions in the optimised fermented beverage

Based on the results of the BBD model, the optimised fermented broccoli-leaf beverage was selected, and two different processing treatments were studied: high-temperature short-time (HTST) pasteurisation and HHP. For HTST, 500 mL of fermented beverage was heated in a stainless-steel container at 72 ± 2 °C for 15 s (Rios-Corripio et al., 2022), followed by rapid cooling with a stream of cold-water. This treatment was carried out in a Mastia thermoresistometer (Conesa et al., 2009) and the treated beverage was bottled in a 20 mL polyethylene terephthalate (PET) bottle. For the HHP treatment, the fermented beverage was bottled in a 20 mL PET container and submitted to 200 and 400 MPa for 10 and 1 min, respectively, using the High Pressure Pilot Food Processor (Stansted Fluid Power Ltd, Essex, UK). The pressure and time parameters were chosen based on previous studies in fermented beverages (Pega et al., 2018). As a control, the beverage was bottled without any thermal or HHP treatment. The quality was evaluated weekly for a shelf-life of 49 days at 5 ± 1 °C for all treatments. Three replicates were used for each treatment and sampling day.

2.6. Microbiological analysis, TPC, and TEAC

Microbiological analysis was conducted following standard methods. LAB enumeration was performed using MRS Agar at 30 °C for 48 h; mesophilic bacteria was performed using Plate Count Agar (PCA) at 30 °C for 48 h; moulds and yeast counts were obtained using Rose Bengal (RB) and Chloramphenicol at 25 °C for 72 h; and Enterobacteriaceae enumeration was performed using Violet Red Bile Glucose Agar (VRBGA) at 37 °C for 24 h (Scharlau Chemie S.A. Barcelona, Spain). The results were expressed as log CFU/g of fresh or fermented broccoli leaves, or log CFU/mL in fermented beverages.

The TPC and TEAC were measured using a multiscan plate reader (Tecan infinite M200, Männedorf, Switzerland). For the TEAC assay, the ABTS reagent (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) was used (Sigma Aldrich, St. Louis, USA). The measurements were conducted following the method described by Salas-Millán et al. (2022). The results were expressed as mg gallic acid equivalent (GAE) for TPC and mmol of Trolox equivalent (TE) per g of dry weight (DW) in fresh or fermented broccoli leaves or per litre in fermented beverages.

2.7. Identification and quantification of phytochemicals by LC-MS analysis

For the identification and quantification of (poly)phenolic compounds, the extraction procedure was carried out according to Gonzales et al. (2015). After centrifugation and filtration (0.45 μm polyamide membrane filter), the samples were loaded (4 mL) onto a solid-phase extraction (SPE) C18 column (500 mg, 3 mL), previously preconditioned with MeOH. The column was then washed with water and eluted with MeOH. The eluent obtained was dried with nitrogen gas, and re-dissolved in 1 mL of MeOH:H_2O (10:90, 0.1% formic acid).

The (poly)phenolic compounds were identified using a high-pressure liquid chromatograph (HPLC) Agilent 1290 Infinity II LC (Santa Clara, CA, USA) equipped with a G7104A quaternary pump, G7167B Standard Autosampler, G7116B column heater, and coupled to a model 6546 hybrid quadrupole-time-of-flight mass spectrometer (Q-TOF) with a Dual Agilent Jet Stream Ionisation source being used for that purpose. The compounds were separated on a reverse-phase column ZORBAX Eclipse Plus C18 (3.0 × 100 mm; 1.8 μm). The mobile phase was composed of two solvents: A, which was 0.1% formic acid in water, and B, which was 0.1% formic acid in acetonitrile. The analysis was performed in negative mode, and the drying gas flow (N_2) was set at 13 L/min, the nebuliser pressure at 40 psi, the gas drying temperature was 350 °C, and the sheath gas flow and temperature were 12 L/min and 350 °C, respectively. The instrument was set to negative ion mode with an m/z range of 50–1700, a capillary voltage of 4000 V and a fragmentor of 200 V. Data analysis was performed in MassHunter Workstation software. For the tentative identification of individual (poly)phenolic compounds, mass spectrometry (MS) and tandem mass spectrometry (MS^2) fragmentation were used. The fragmentation pattern obtained was compared with available bibliographic data, as well as with the MassBank Europe and Human Metabolome databases.

Quantification of the (poly)phenolic compounds was carried out using an Agilent 1200 liquid chromatography (Santa Clara, CA, USA) according to Salas-Millán et al. (2022). The quantification of hydroxycinnamic acid derivatives was expressed as mg of chlorogenic acid equivalents, and as mg of rutin for flavonoids derivatives, in both cases per litre of fermented beverage.

The quantification of sulforaphane, indol-3-carbinol, and ascorbigen was carried out as per Hauder et al. (2011) albeit with slight differences. The HPLC/MS equipment, column, and mobile phases used for quantifying (poly)phenols were also used for the quantification of the organosulfur compounds. The analysis was performed in positive mode, and the mass spectrometer parameters were set as follows: a capillary voltage of 3500 V, a gas temperature of 325 °C, a gas flow rate of 10 L/min, and a nebuliser pressure of 45 psi. Only one transition was considered for the quantification: m/z 178.1 → 114.1 for sulforaphane, m/z 130.1 → 77.3 for indol-3-carbinol, and m/z 306.1 → 130.2 for ascorbigen. Organosulfur compounds were quantified using authentic standards and expressed as mg L^{-1} in the fermented beverage.

2.8. Inhibition of α-glucosidase and α-amylase

The α-glucosidase inhibitory activity was evaluated following Gong et al. (2020) with minor differences. The assay was conducted using a multiscan plate reader (Tecan infinite M200, Männedorf, Switzerland) and α-glucosidase from *Saccharomyces cerevisiae* Type I (Sigma Aldrich, St. Louis, USA). The inhibition percentage (I, %) was calculated according to the following equation:

$$I\,(\%) = \left(1 - \frac{v_1}{v_0}\right) \times 100$$

where v_0 and v_1 are the initial reaction velocity without and with the presence of the fermented beverage, respectively.

For the assessment of α-amylase inhibitory activity (Johnson et al.,

2011), Type VI-B porcine pancreas (Sigma Aldrich, St. Louis, USA) was used, and the inhibition percentage (I, %) was calculated as follows:

$$I\ (\%) = \left(1 - \frac{A_{sample} - A_{blank-sample}}{A_{control} - A_{blank-control}}\right) \times 100$$

where A_{sample} and $A_{control}$ are the absorbance values of the sample and control, and $A_{blank\text{-}sample}$ and $A_{blank\text{-}control}$ are the blank sample and control, respectively.

2.9. Statistical analysis and experimental design for optimisation

Section 2.4 described the optimisation parameters of the fermentation–assisted extraction of broccoli leaves using the Box-Behnken design. A three-factor, three-level model was constructed, and the second-order polynomial coefficients were calculated and analysed using Statgraphics Centurion XV (Statgraphics Technologies, Inc, The Plains, Virginia, USA). This software was used to estimate the effects of the variables on the final response, the second-order mathematical model, Pareto charts, the optimum levels of the significant variables, and the response surface graphs. The profile of the predicted values and the desirability analysis were used on a scale ranging from 0.0 (undesirable) to 1.0 (highly desirable).

To determine the effect of processing treatments (HTST pasteurisation and HHP) and storage conditions (49 days at 5 °C) on the optimised fermented beverage, a two-way analysis of variance (ANOVA) was conducted. Mean values were compared using LSD (multiple range least significant difference test) to identify significant differences among factors and interactions between them. A hierarchical heatmap was produced for each treatment using MetaboAnalys 5.0 (www.metaboanalys.ca). The clustering employed Ward's method, with Euclidean algorithms determining the distances between data points. Furthermore, the Pearson's correlation coefficient (ρ) was calculated to evaluate the correlation in the evolution of the phytochemical with time.

3. Results and discussion

3.1. Obtaining optimised fermented beverage through Box-Behnken design (BBD)

The three-level BBD design used to investigate the optimal fermentation conditions to obtain the highest TPC and TEAC concentrations and LAB levels in the fermented beverage derived from broccoli leaves is shown in Table 1. The experimental and predicted values of the responses (TPC, TEAC and LAB) are also shown therein. Table 2 shows the ANOVA results for the factors affecting TPC, TEAC, and LAB responses. The table includes the sums of squares and the probability of significance ($p < 0.05$; values in bold) for the linear effects of the responses (X_1: time, X_2: solid-solvent ratio, and X_3: temperature), the quadratic expressions (X_1X_1, X_2X_2, and X_3X_3), and the interaction effects of the factors (X_1X_2, X_1X_3, and X_2X_3). The final model (Table 1) is presented for each response with its corresponding constant and coefficients, which was fitted using the significant variables to obtain appropriate prediction equations (Susanti et al., 2022).

The Pareto chart reveals that the sign of certain linear effects differs (Fig. 1). This divergence in the linear effect is attributed to the influence of multicollinearity, where multivariate relationships align more accurately with the underlying truth. However, the effects on the responses, whether negative or positive, only consider the relationships per factor in the Pareto chart (McElreath, 2018). As shown in Table 2, the non-significant *p*-value of lack-of-fit test demonstrated that the predicted model fits the observed data well at the 95.0% confidence level. The parity plots (Fig. 1) demonstrate a high adjusted correlation coefficient value (R^2-adj); this is more suitable for comparing models with different numbers of independent variables and indicates a strong correlation between predicted and observed data. All plots have an R^2-adj value greater than 0.75, indicating a satisfactory model fit according to the literature (Le Man et al., 2010).

The response to LAB counts showed that only temperature had a significant negative effect. This was the case for the temperature range studied (25–37 °C) because the specific growth rate and lag phase duration of LAB are temperature dependent (Matejčeková et al., 2019), and increase with the temperature.

The extraction of phenolic compounds from broccoli leaves during the fermentative process was found to be affected by the solid-solvent ratio, temperature, and time, as indicated by their low *p*-values. Significant interactions were observed between time with the solid-solvent ratio, time with temperature, and the quadratic effect of time (Table 2). From the Pareto chart, it is recommended to increase the solid-solvent ratio and extend the fermentation time to enhance the extraction of phenolic compounds from the broccoli leaves during the fermentative process. The significant interactions mentioned had a positive impact on TPC extraction. These findings are consistent with those of other researchers (Jaiswal & Abu-Ghannam, 2016). However, the linear response of temperature had a negative effect on TPC extraction, as reported by Adebo et al. (2018). The TEAC was only affected by the three significant linear variables ($p < 0.05$). The fermentation time and solid-solvent ratio had a positive effect on the antioxidant capacity of the fermented beverage (Fig. 1). In contrast, temperature had a negative effect on TEAC. The similarities in the effects on the TPC and TEAC responses are based on the relationship between the content of these phytochemicals and their antioxidant capacity (Deng et al., 2013).

This study provides insights into the factors affecting TPC and TEAC concentrations, as well as LAB growth. However, to optimise fermentation-assisted extraction for broccoli leaves with *L. plantarum*, a

Table 2
Analysis of variance for the independent variables in fermented-assisted extraction broccoli-leaf beverage.

Source	LAB (log CFU/mL)		TPC (mg GAE/L)		TEAC (mmol TE/L)	
	Sum of squares	p > F	Sum of squares	p > F	Sum of squares	p > F
X_1: Time	0.01795	0.6209	3293.85	**0.0082**	0.01152	**0.0267**
X_2: Solid-solvent ratio	0.02360	0.5749	34275.5	**0.0008**	0.12124	**0.0026**
X_3: Temperature	1.21641	**0.0413**	5583.61	**0.0049**	0.05876	**0.0054**
X_1X_1	0.12009	0.2728	515.658	**0.0493**	0.00030	0.4352
X_1X_2	0.02620	0.5564	1092.21	**0.0242**	0.00563	0.0525
X_1X_3	0.00051	0.9314	521.045	**0.0488**	0.00024	0.4803
X_2X_2	0.19619	0.1956	355.639	0.0692	0.00193	0.1335
X_2X_3	0.00233	0.8539	236.472	0.099	0.00050	0.3368
X_3X_3	0.78635	0.0618	136.677	0.1552	0.00204	0.1278
Lack of fit	0.01398	0.9607	988.241	0.0778	0.00458	0.1784
Pure error	0.10698		54.8161		0.00064	
Core total	2.53266		47073.2		0.20771	

Codification of independent ranged factors: X_1, time in days; X_2, solid-solvent ratio as g of broccoli leaves per mL of water (w/v); X_3, temperature in °C. TPC: Total phenolic content; TEAC: Trolox equivalent antioxidant capacity; LAB: Lactic acid bacteria count. Values in bold are significant at $p < 0.05$.

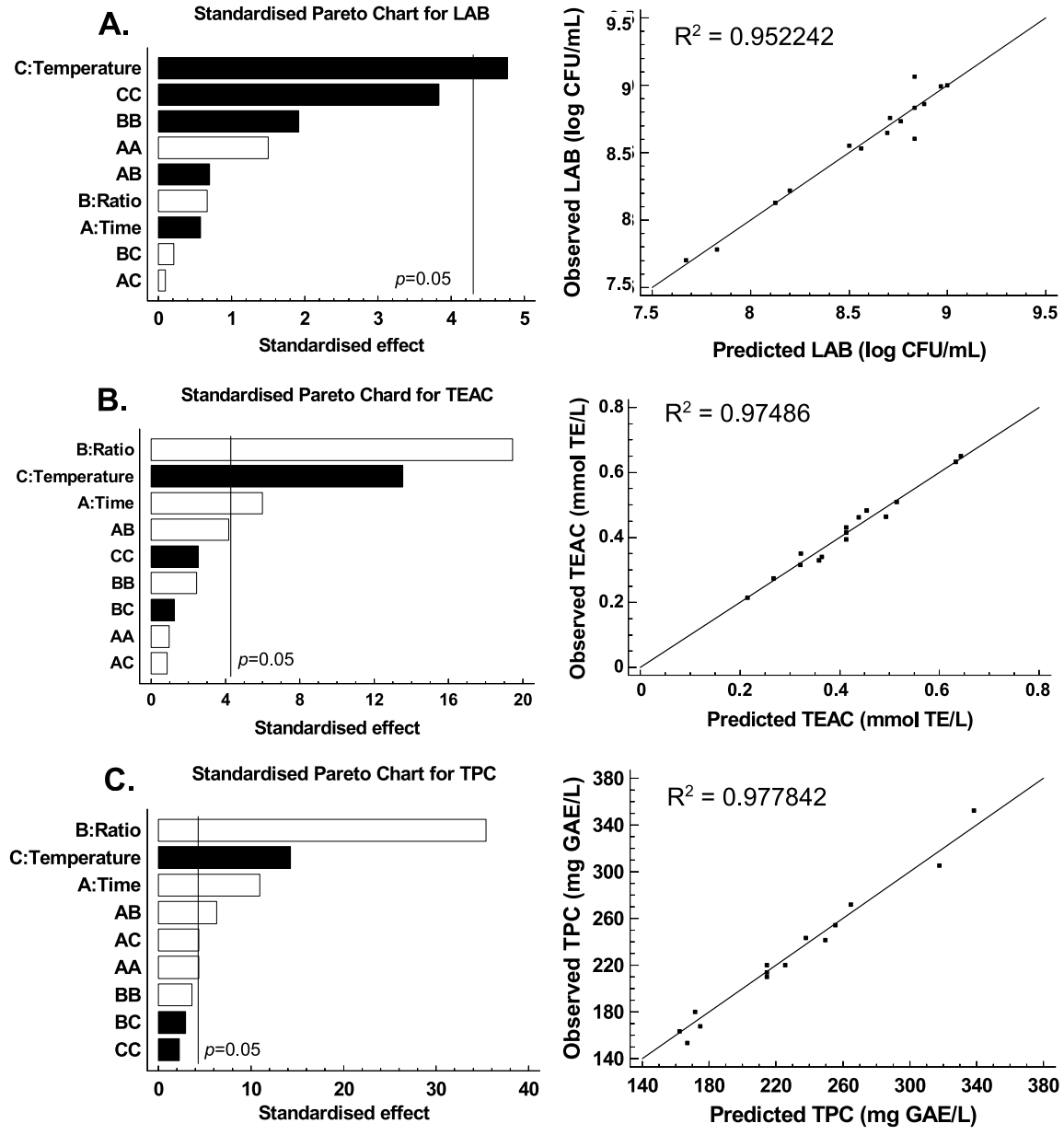

Fig. 1. Pareto chart for standardised effects of factors in Box-Behnken experimental design and parity plot to show the distribution of predicted and observed values for each experimental response with coefficient-regression, in fermented-assisted extraction broccoli-leaf beverage: (A) LAB: Lactic acid bacteria count, (log CFU/mL) (B) TPC: Total phenolic compounds (mg GAE/L); (C) TEAC: Trolox Equivalent Antioxidant capacity (mmol TE/L). Factors with a significant effect ($p < 0.05$) are crossed by the blue line. □ Indicates a positive effect and ■ indicates a negative effect.

multi-response optimisation analysis is required in order to determine the optimal value for each factor.

3.2. Multi-response optimisation of fermentation process for broccoli-leaf beverage

The optimal values for the factors were determined using the desirability function (Fig. 2), which ranges between $D = 0$ (representing an unacceptable response) to $D = 1$ (indicating a completely desirable one). Individual desirability was determined in the previous section, and the predicted values obtained were transformed into a dimensionless scale (d_i), where i represents an individual response. D was calculated by combining the individual desirability values using a geometric mean: $D = d_{TPC} \times d_{TEAC} \times d_{LAB}$. The D function was then subjected to an algorithm to find the set of variable values that maximise it. This function has been widely used in the optimisation of analytical systems with multiple responses (Ferreira et al., 2007). The optimal conditions for the fermentation–assisted extraction were found to be four days, a solid-solvent ratio of 0.1 (w/v), and a temperature of 27.77 °C, resulting in an optimal D value of *0.955*.

3.3. Processing treatments and storage conditions for optimised fermented broccoli-leaf beverage

3.3.1. Microbiological analysis

The initial phytobiome of the fresh broccoli leaves presented 2.40 log CFU/g for LAB, 4.89 log CFU/g for mesophiles and 3.31 log CFU/g for Enterobacteriaceae. Mould and yeast were presented at 2.20 and 2.82

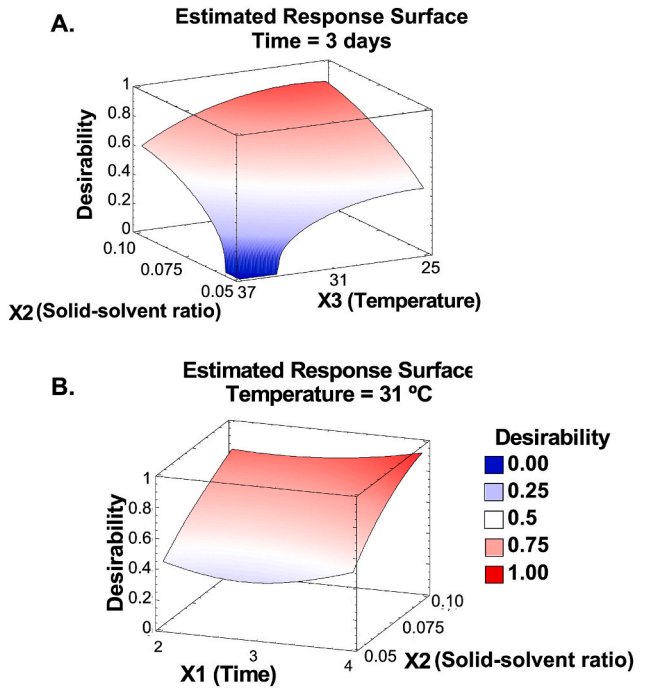

Fig. 2. Response surface graphs with respect to desirability response (0–1). A: Temperature (°C) vs. solid-solvent ratio (g of broccoli leaves per mL of water). B: Solid-solvent ratio vs. time of fermentation (days).

Table 3
Microbiological analysis, total phenolic content (TPC), and Trolox equivalent antioxidant capacity (TEAC) in fresh and fermented broccoli leaves.

Leaves	Fresh	Fermented
LAB	2.40 ± 0.31	8.36 ± 0.14
MES	4.89 ± 0.13	8.38 ± 0.10
Moulds	2.20 ± 0.20	<1
Yeast	2.82 ± 0.15	2.00 ± 0.20
Enterobacteriaceae	3.31 ± 0.25	2.17 ± 0.17
TPC	205.11 ± 1.30	94.59 ± 1.18
TEAC	0.39 ± 0.03	0.11 ± 0.01

Mean (n = 3) ± SE. TPC (mg GAE/g DW). TEAC (mmol TE/g DW). Microbiological analysis of lactic acid bacteria (LAB), mesophiles (MES), moulds, yeasts, and Enterobacteriaceae as log CFU/g of fresh weight. Fermentative broccoli leaves conditions: 4 days at 27.77 °C with a 0.1 solid-solvent ratio.

log CFU/g, respectively (Table 3). During the fermentation process and inoculation with *L. plantarum* (6 log CFU/mL), the population of moulds, yeasts, and Enterobacteriaceae in the broccoli leaves dropped to <1, 2.00, and 2.17 log CFU/g, respectively. That microbial reduction obtained during fermentation is attributed to the presence of *L. plantarum,* which produces lactic acid, lowering the pH (pH < 4). The levels of LAB and mesophilic bacteria in the broccoli leaves increased and were similar at 8.37 log CFU/g at the end of fermentation (Table 3). This similarity between both bacterial groups is due to *L. plantarum* growing under mesophilic conditions (Ko et al., 2023). Therefore, the results and discussion regarding these populations are comparable without any significant differences.

Once the fermented beverage had been obtained, the processing treatments (HTST pasteurisation and HHP) and storage conditions (49 days at 5 °C) were evaluated (Table 4). Moulds, yeasts, and Enterobacteriaceae populations remained stable and below the detection limit (<1 log CFU/mL) throughout the study (data not shown). The processing treatment was the primary factor ($p < 0.001$) contributing to 89% of the total variance (PVT) for LAB and mesophilic bacteria. Although the storage time had a statistically significant impact ($p < 0.001$), its contribution to PTV was low, at 2.53% and 2.65% for LAB and mesophilic bacteria, respectively (Table 4). The beverage treated with both HHP at 200 MPa and the control (untreated) had the highest LAB levels (<8 log CFU/mL), with the population decreasing to 6.6 log CFU/mL over 49 days (Fig. 3). However, both HTST pasteurisation and HHP at 400 MPa significantly reduced microbial counts to an average of 3.3 and 2.4 log CFU/g, respectively. After 49 days at 5 °C, the populations of LAB and mesophiles decreased, resulting in average final populations of 2.3 and 1.5 CFU/mL, respectively (Fig. 3). The levels of mesophiles followed a similar trend to those of the LAB levels, as previously stated. Yang et al. (2021) reported the survival rate of *L. plantarum* under HHP treatments and the pressure- and time-dependence of the process. These results are consistent with our LAB values and the pressure-dependent survival rates of *L. plantarum.* In contrast, thermal treatments are known to affect microbial viability and enzymes (Rios-Corripio et al., 2020) and, as expected, the HTST treatment reduced the microbial count. This would generally be a desirable effect, but the aim was to obtain a fermented drink that is rich in LAB for a probiotic effect.

These results highlight promising opportunities for enhancing the safety and stability of fermented beverages through non-thermal treatments such as HHP under low pressure. Furthermore, the high LAB levels observed in the control treatment, without any processing treatments, suggest that fermentation processes can enhance food stability and prolong shelf-life. This method is of particular interest since it is sustainable, uses minimal energy consumption and relies on the natural metabolism of LAB to stabilise food during long-term storage.

3.3.2. TPC and TEAC

The initial TPC in the broccoli leaves was 205.1 mg GAE/g DW, which decreased to 94.60 mg GAE/g DW after the fermentation (Table 3). A similar trend was observed for the antioxidant capacity, with TEAC decreasing from 0.39 to 0.11 mmol TE/g DW. This study suggests that phenolic compounds were transferred from the broccoli leaves to the beverage during the fermentation process, as reported by Salas-Millán et al. (2022) in their study of fermented broccoli by-products (stalks). The fermentation process affects the cell wall of the broccoli leaves, which facilitates the release of these phytochemicals into the fermented beverage (Hur et al., 2014).

Regarding the processing treatments and storage conditions of the fermented beverage, the PTV for TPC was primarily influenced by storage time and processing treatment, accounting for 58.29% and 63.51%, respectively. Statistical analysis showed a significant difference ($p < 0.001$) for these factors and their interaction, which accounted for 12.37% of the PTV (Table 4). HTST pasteurisation increased the TPC from 222.4 mg GAE/L (control fermented beverage) to 295.9 mg GAE/L (Fig. 3), which decreased during the shelf-life. Some authors have described this phenomenon as a kinetic transfer resulting from an increase in temperature in fruit pulp and juice (Salar et al., 2021; Wang et al., 2020). For the HHP treatments, both 200 and 400 MPa kept the TPC values lower than the control and HTST treatments. However, with HHP at 200 MPa the TPC remained more stable throughout the shelf-life. This concurs with Liu et al. (2019), who also found a decrease in flavonoids after HHP treatment at 300 and 500 MPa in infusions of green tea leaves. Moreover, some authors reported a negative effect upon increasing the pressure in fruit smoothies (Keenan et al., 2012). The effects of both time and temperature on hydrostatic pressure on food varies and depends on the food matrix (Ahmed & Eun, 2018).

For antioxidant activity, the PTV for TEAC was mainly influenced by the processing treatment and storage time, accounting for 42.71% and 14.97%, respectively. Statistical analysis indicated a significant difference ($p < 0.001$) for these factors, although the interaction was not significant it accounted for 30.69% of the PTV (Table 4). TEAC followed a similar trend to TPC, with the beverage under HTST pasteurisation showing the highest TEAC, followed by the control and HHP treatments. Once again, the HHP treatment at 200 MPa resulted in a beverage with a slightly higher TEAC (0.30–0.29 mmol TE/L) compared to that at 400

Table 4
Microbiological analysis, total polyphenolic content (TPC), and Trolox equivalent antioxidant capacity (TEAC) in the optimised fermented broccoli-leaf beverages after thermal, HPP (200 and 400 MPa), and control treatments, stored for 49 days at 5 °C.

Source of variable	LAB		MES		TPC		TEAC	
	PTV	LSD	PTV	LSD	PTV	LSD	PTV	LSD
Processing treatment (T)	89.71%	(1.00)***	89.66%	(0.98)***	58.29%	(4.19)***	42.71%	(0.24)***
Time days (D)	2.53%	(0.71)***	2.65%	(0.69)***	63.51%	(2.95)***	14.97%	(0.17)***
T × D	0.65%	(1.41)^{ns}	0.64%	(0.75)^{ns}	12.37%	(5.89)***	30.69%	(0.19)**
Means								
Processing treatment	**log CFU/mL**		**log CFU/mL**		**mg GAE/L**		**mmol TE/L**	
Control	7.46 ± 0.22^{a}		7.40 ± 0.22^{a}		248.0 ± 6.5^{b}		0.36 ± 0.01^{b}	
HTST	2.94 ± 0.13^{b}		3.10 ± 0.07^{b}		263.8 ± 5.8^{a}		0.44 ± 0.02^{a}	
HHP 200	7.35 ± 0.19^{a}		7.33 ± 0.21^{a}		219.2 ± 2.4^{c}		0.31 ± 0.01^{c}	
HHP 400	1.92 ± 0.16^{c}		2.10 ± 0.15^{c}		200.7 ± 4.6^{d}		0.29 ± 0.02^{c}	
Time - days								
0	5.54 ± 1.54^{a}		5.60 ± 1.52^{a}		234.7 ± 3.1^{abc}		0.36 ± 0.00^{b}	
7	5.29 ± 1.62^{a}		5.45 ± 1.54^{a}		231.9 ± 0.5^{a}		0.42 ± 0.01^{a}	
14	5.14 ± 1.57^{bc}		5.19 ± 1.49^{ab}		232.6 ± 2.2^{ab}		0.38 ± 0.00^{ab}	
21	5.26 ± 1.34^{ab}		5.24 ± 1.34^{ab}		231.5 ± 2.5^{bc}		0.36 ± 0.00^{b}	
28	4.72 ± 1.40^{bcd}		4.76 ± 1.33^{bc}		234.7 ± 1.7^{abc}		0.34 ± 0.01^{bc}	
35	4.68 ± 1.36^{cd}		4.63 ± 1.30^{c}		238.7 ± 1.5^{abc}		0.31 ± 0.01^{c}	
42	4.43 ± 1.42^{d}		4.60 ± 1.32^{c}		226.0 ± 0.8^{c}		0.31 ± 0.01^{c}	
49	4.29 ± 1.38^{d}		4.39 ± 1.31^{c}		233.0 ± 1.9^{abc}		0.31 ± 0.00^{c}	

Means (n = 3) ± SE. Control: Not treated. High-Temperature Short-Time (HTST) pasteurisation: 72 ± 2 °C for 15 s. High-hydrostatic pressure (HHP) treatments: 200 MPa for 10 min and 400 MPa for 1 min. Mean values were compared for each factor using the LSD (multiple range least significant difference test) at $p < 0.001$ (***). The percentage of total variance (PTV) is also shown.

MPa (0.30–0.23 mmol TE/L). In all treatments, there was a general reduction in TEAC observed with storage time. The differences shown between the TPC and TEAC measurements are due to the lack of an exact correlation between these values. According to Deng et al. (2013), phenolic compounds are not solely responsible for the antioxidant capacity, owing to differences in reactivity with the Folin reagent and the different phenolic compounds (Singleton et al., 1999). Conducting an accurate analysis and identification of each compound using liquid chromatography coupled with mass spectrometry would be beneficial in order to understand the trends and transformations of these individual phytochemicals during processing treatments and shelf-life, as reported in the following section.

3.4. Identification of (poly)phenolic compounds in optimised fermented broccoli-leaf beverage

Fifteen total (poly)phenols were detected, six of which are classified as phenolic acids whilst the remaining nine other compounds are flavonoids. The exact molecular ion mass, and main fragment ions are shown in Table 5. The flavonoids were further classified into deacylated and acylated flavonoids, depending on their chemical side chain. This identification is discussed in Supplementary Section (Figs. S1–S3).

3.5. Phytochemical changes during processing treatments and storage conditions for optimised fermented broccoli-leaf beverage

The changes in the individual phytochemical compounds according to the type of heat treatment and storage time were analysed using a hierarchical heatmap (Fig. 4A). The intensity of the red or blue colour indicated higher or lower relative quantification of phytochemical compounds, respectively. Additionally, Pearson's correlation coefficient (ρ) plots showed the negative ($\rho < 0$) and positive ($\rho > 0$) correlation of phytochemical content for each treatment (Fig. 4B, C, D, and E).

The resulting clusters, based on the mean values, showed two different groups: one included the beverage from the control, HTST, and 200 MPa HHP treatments, whilst the second group was represented by the beverage treated with 400 MPa HHP. This main cluster division was due to the lowest concentration of phytochemicals after the HHP treatment at 400 MPa, particularly in organosulfur compounds and flavonoids such as **dK2**, **aK1**, and **aK3**.

Nevertheless, the 400 MPa HHP treatment showed better stabilisation of phenolic acids and flavonoids during refrigeration, with $\rho > 0$ (Fig. 4E) for 49 days. On the other hand, after the control treatment, chlorogenic acid isomers (mainly **3**- and **5CQa**) decreased during storage ($\rho < -0.6$). The HHP treatments showed a better stabilisation ($\rho \approx 0$) in these chlorogenic acid isomers, followed by the HTST treatment, which showed a smaller decrease ($\rho \geq -0.5$). This suggests that certain factors affect the stability of these compounds, which is inhibited after HTST pasteurisation and HHP treatments. Polyphenol oxidase (PPO) activity is known to be responsible for the enzymatic browning reaction, degradation, and oxidation of monophenols and o-diphenols (Gawlik-Dziki et al., 2007; Westphal et al., 2017). PPO is sensitive to thermal and HHP treatments, and its activity decreased (Iqbal et al., 2019). Moreover, the metabolic pathways present in *Lactobacillus* spp. strains involve glycosyl hydrolases that convert flavonoid glycosides into their respective aglycones. In addition, esterases are involved in the breakdown of methyl gallate, tannins, and esters of phenolic acids (Filannino et al., 2015; Gaur & Gänzle, 2023). These esterases can hydrolyse the ester linkage between phenolic acid and flavonoid in acylated flavonoid glycosylates. This was observed by the decrease in **aK1** and **aK3** and the corresponding increase in **dK2** in the control treatment, which is a result of the cleavage of these acylated bonds.

The hierarchical heatmap sub-grouped the control and 200 MPa HHP treatments together, indicating similar trends in the phytochemical composition. Both treatments showed the highest content of indolic compounds (indole-3-carbinol and ascorbigen). However, the HHP 200 MPa treatment stood out with the highest concentration in sulforaphane, 8.82 to 4.38 mg/L (Table S1). Pearson's Correlations plots highlighted the similar degradation of indolic compounds among the organosulfur compounds for all the treatments, as shown by Pearson's Correlations plots. Ciska et al. (2021) reported different results, describing a stability of indolic compounds (ascorbigen) and a decrease in isothiocyanates during the storage of sauerkraut juice. In contrast, Cai et al. (2019) reported a stabilisation of sulforaphane after 20 days of fermentation, followed by a decrease for 90 days. These results demonstrate the potential for extracting bioactive compounds from

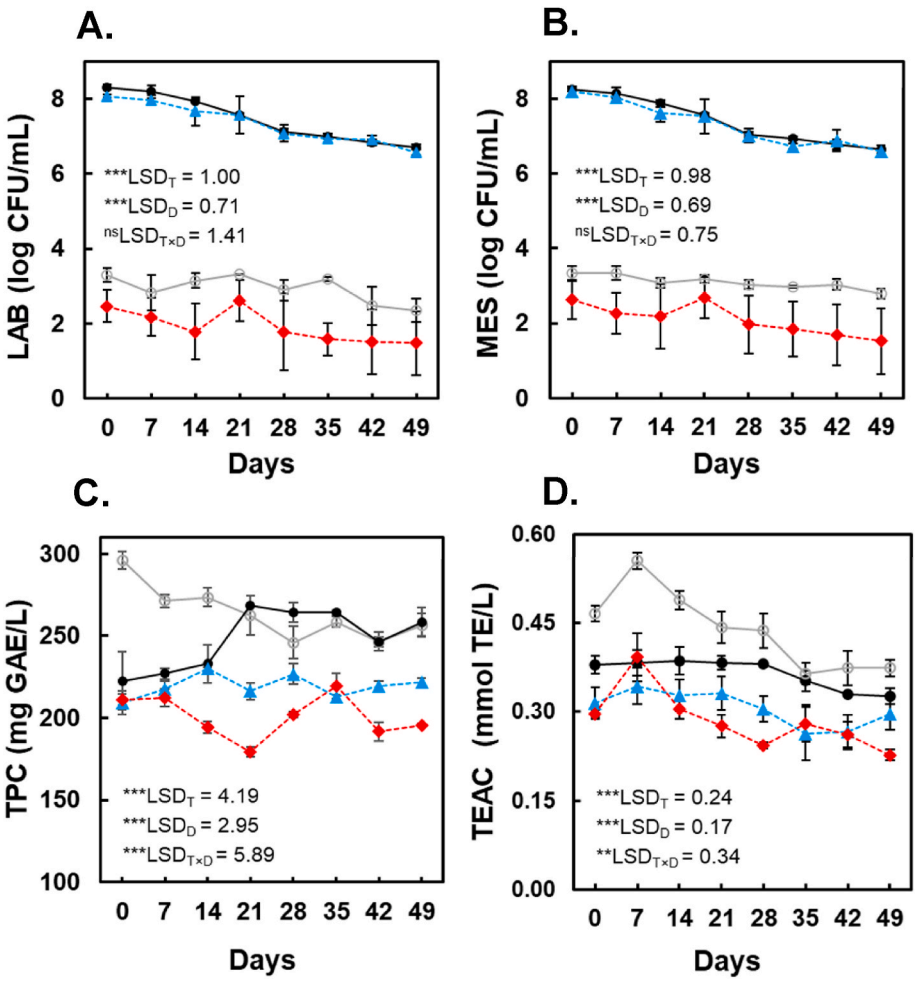

Fig. 3. Lactic acid bacteria (A), mesophilic bacteria (B), total phenolic compounds (C), Trolox equivalent antioxidant capacity (D) of optimised fermented broccoli-leaf beverage after thermal (○ HTST pasteurisation), high hydrostatic pressure-HHP (▲ 200 MPa HHP, and ◆ 400 MPa HHP), and (●) control treatments during shelf-life at 5 °C. Data represent means of three replicates (n = 3 ± SE). LSD (0.1%)***; LSD(1%)**; no significant difference (^{ns}). T: processing treatment. D: time in days.

Table 5
List of putatively (poly)phenolic compounds identified in the optimised fermented broccoli-leaf beverage.

ID	Tentative identification	Molecular formula	Molecular weight	m/z experimental	Error (ppm)	MS^2 fragments
	Phenolic acid					
3CQa	3-O-Caffeoylquinic acid	C16H18O9	354	353.0882	1.06	191, 179, 135
5Cqa	5-O-Caffeoylquinic acid	C16H18O9	354	353.0886	2.25	191
4Cqa	4-O-Caffeoylquinic acid	C16H18O9	354	353.0876	−0.58	191, 173, 179
1Fqa	1-O-Feruloylquinic acid	C17H20O9	368	367.1042	2.05	193, 134, 173
5Fqa	5-O-Feruloylquinic acid	C17H20O9	368	367.1032	8.29	191, 173, 193
S1	1,2-Disinapoylgentiobioside	C34H42O19	754	753.2322	9.89	529, 205, 223
	Deacylated flavonoids					
dK1	K-3,7-O-diGlu	C27H30O16	610	609.1472	1.79	285, 447
dK2	K-3-O-Soph-7-O-D-Glu	C33H40O21	772	771.1984	−0.68	771, 609, 446
dK3	K-3-O-sophoroside	C27H30O16	610	609.1456	−0.83	283, 446
dK4	K 3-O-Sophtrioside-7-O-Soph	C45H60O31	1096	1095.3959	−7.92	933, 686, 466
dQ1	Q-diGlu	C27H30O17	626	625.1421	1.72	300
	Acylated flavonoids					
aK1	K 3-O-hFer-Soph-7-O-Glu	C45H56O23	964	963.2409	−0.3	801, 609, 285
aK2	K 3-O-Caf-Soph-7-O-Glu	C42H46O24	934	933.2305	−0.13	771, 609, 286, 429
aK3	K-3-O-Sin-Soph-7-O-Glu	C44H50O25	978	977.2563	−0.55	815, 609, 446
aK4	K-3-O-FerSoph-7-O-Glu	C43H48O24	948	947.2455	−0.82	785, 609, 446, 284

Kaempferol (K); Quercetin (Q); Glucoside (Glu); Sophoroside (Soph); Sinapoyl (Sin); Feruloyl (Fer); Hydroxyferuloyl (hFe); Caffeoyl (Caf).

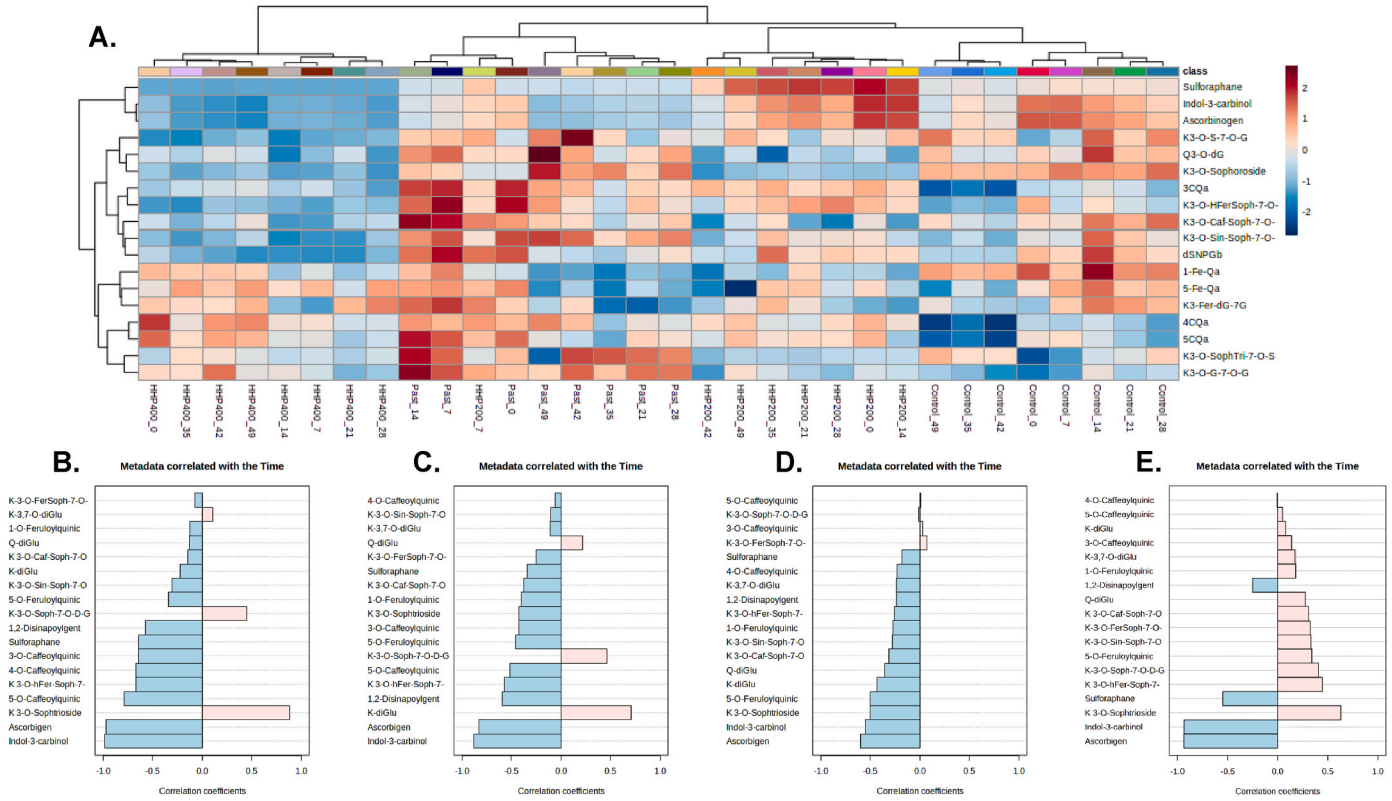

Fig. 4. Heatmap graph of changes and distribution of phytochemical of optimised fermented broccoli-leaf beverage (A), and Pearson's Correlation Coefficient graphs for time of storage (49 days at 5 °C) for control (B), HTST pasteurisation (C), HHP 200 MPa (D) and HHP 400 MPa (E) treatments. Kaempferol (K); Quercetin (Q); Glucoside (Glu); Sophoroside (Soph); Sinapoyl (Sin); Feruloyl (Fer); Hydroxyferuloyl (hFe); Caffeoyl (Caf).

broccoli by-products through bioprocesses such as fermentation by *L. plantarum,* as previously reported by Salas-Millán et al. (2023). Furthermore, the stability and transformation of these phytochemicals after HHP treatments enhance our comprehension of the dynamics of compounds during food processing and storage, particularly in fermented beverages.

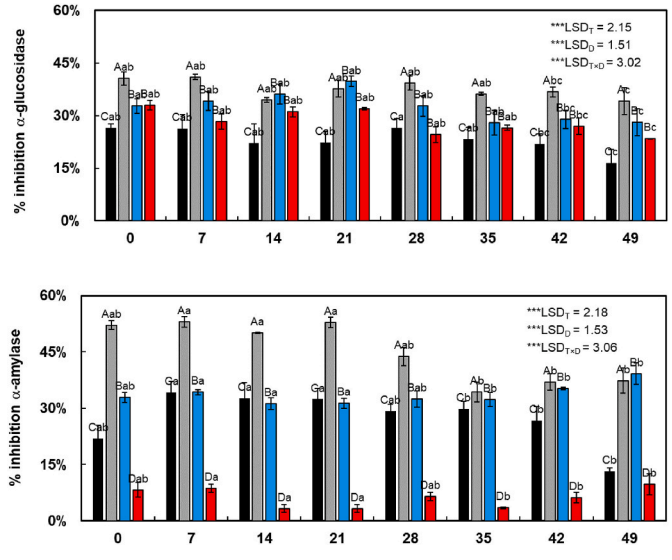

Fig. 5. Changes in percentage of inhibition rate in α-glucosidase (A) and α-amylase (B) of optimised fermented broccoli-leaf beverage under control (■), HTST pasteurisation (■), 200 MPa (■) and 400 MPa high-hydrostatic pressure (■) treatments. Data represent means of three replicates (n = 3 ± SE). Different letters indicate significant differences according to LSD (0.1%) (capital letters for processing treatments-T and lower-case letters for days of storage -D).

3.6. *Inhibitory effect on α-amylase and α-glucosidase*

The percentage of inhibition for α-glucosidase and α-amylase was measured after the processing treatments and during the shelf-life (Fig. 5). The fermented beverages treated with HTST pasteurisation showed the highest inhibition percentages for both digestive enzymes, ranging from 34% to 52%, followed by the control and HHP 200 MPa and 400 MPa treatments. The differences in the (poly)phenolic profile were mainly related to the content of phenolic acids, especially in **3CQa** and **5CQa**. Aleixandre et al. (2022) reported that chlorogenic acid had an IC50 (half maximal inhibitory concentration) of 1380 mg/L and 1130 mg/L for α-amylase and α-glucosidase inhibition, respectively. The sum of both CQa isomers in the HTST treated beverage was less than 400 mg/L throughout the storage time, and the inhibition value for both enzymes was higher than expected. This was due to the presence of other phenolic acids and flavonoids that contribute to this antidiabetic bioactivity (Lo Piparo et al., 2008).

A low inhibition activity for both enzymes in the control treatment compared to the HTST treatment was correlated with a lower content of total individual phenolic acids (280 mg/L to 172 mg/L). Thus, the content of deacylated and acylated flavonoids remained stable at around 170 mg/L and 115 mg/L, respectively. This suggests that the presence of phenolic acids, particularly **3CQa** (3-O-Caffeoylquinic acid), was the main contributor to the inhibition of α-amylase and α-glucosidase activity.

The fermented beverage treated with 200 MPa HHP maintained α-glucosidase and α-amylase inhibition values of between 33 and 40% throughout storage. The increase in the α-amylase inhibition rate over time is related to the increase in deacylated flavonoids, mainly in **dK3** (kaempferol di-glucoside), which increased from 76 to 101 mg/L

(Table S1) after 49 days of cold storage. However, this effect was not observed in the α-glucosidase inhibition due to the difference in kaempferol derivatives responses between enzymes (Costa Silva et al., 2019).

Finally, the fermented beverage treated with 400 MPa HHP showed significant differences in inhibition activity between α-glucosidase and α-amylase. This treatment resulted in the lowest (poly)phenol content, mainly in deacylated flavonoids, with a total of 81.3–104.9 mg/L for this flavonoid derivative throughout the cold storage. Kaempferol derivatives have a higher inhibition potential for α-glucosidase than α-amylase (Costa Silva et al., 2019; Yin et al., 2018). Thus, flavonoids with a high number of hydroxylation groups, particularly in the carbon C3, C3', and C4', have a greater inhibitory potential in α-glucosidase. This affects the enzyme's sensitivity to these compounds, meaning that a decrease in their concentration leads to a decrease in α-amylase inhibitory activity compared to other treatments, as reported by Huang et al. (2021).

Considering these results, although HTST pasteurisation showed a higher potential inhibition for α-amylase and α-glucosidase, its decrease was not uniform over time, as in the control treatment. Nevertheless, the 200 MPa HHP treatment had greater uniformity in its antidiabetic potential over refrigeration storage due to its stability in (poly)phenol content.

4. Conclusions

This study presents a method for unlocking the bioactive potential of broccoli leaves by using fermentation-assisted extraction to develop a novel antidiabetic beverage that is lactofermented by *Lactiplantibacillus plantarum*. The fermentation process was optimised using a Box-Behnken design for maximising LAB counts, TPC, and TEAC. The optimisation was achieved after four days of fermentation, with a ratio of 0.1 g of leaf per litre of water and a temperature of 27.77 °C. This optimised fermented broccoli-leaf beverage was then subjected to different processing treatments, including HSTS pasteurisation (72 °C, 15 s), HHP using 200 MPa for 10 min or 400 MPa for 1 min, or non-treatment as a control, and stored for 49 days at 5 °C.

HTST pasteurisation of the beverage resulted in a higher (poly) phenolic content with a LAB population (about 3.5 log CFU/mL), resulting in a loss of probiotic potential. Furthermore, this treatment exhibited the highest percentage of inhibition for both α-amylase and α-glucosidase (ranging from 34% to 52%). The (poly)phenolic content of the control beverage, which did not undergo post-fermentation processing treatment, decreased in chlorogenic acids particularly, also with a decrease in its indole content (ascorbic acid and indol-3-carbinol) as the storage time was extended. Despite this, the LAB population only decreased slightly over time from 8 to 6.5 log CFU/mL, a similar scenario was found after treatment with HHP 200 MPa. Finally, the beverage treated with HHP at 200 MPa maintained a stable content of phytochemicals and antidiabetic potential (ranging from 33% to 40%) and presented the highest content of sulforaphane (4.38–8.82 mg/L) over cold storage. In contrast, the beverage treated with 400 MPa obtained the worst results, with a reduced LAB population (2 log CFU/mL), (poly)phenolic content, and α-amylase inhibition.

In conclusion, the results suggest that the 200 MPa HHP treatment was the most suitable method to achieve stability throughout the shelf-life of the LAB population and phytochemicals. Once an optimal beverage and its post-fermentation treatment have been developed, it is crucial to scale up the process and conduct an organoleptic acceptance study with different flavours and tastes to enhance the acceptability of this innovative functional beverage. This work exemplifies the valorisation of broccoli by-products in accordance with a circular economy model.

CRediT authorship contribution statement

José Ángel Salas-Millán: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Andrés Conesa-Bueno:** Supervision, Conceptualization. **Encarna Aguayo:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

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.

Dra. Encarna Aguayo Giménez.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.103999.

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