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Functional food obtained from fermentation of broccoli by-products (stalk): Metagenomics profile and glucosinolate and phenolic compounds characterization by LC-ESI-QqQ-MS/MS

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ABSTRACT

This research revalues broccoli stalk by-product as a novel fermented health food, using its autochthonous microbiota. Broccoli stalk slices were packed into glass jars, brine was added (6% w/v), and two dressings were studied: garlic, and mustard seeds. No dressing was used as control. Natural fermentation was carried out at 25 °C for 6 days followed by a further 6 days storage at 4 °C. The identification of individual glucosinolates and phenolic compounds was performed by LC-ESI-QqQ-MS/MS. At day 3, the highest content in functional compounds such as glucoerucin and indolic glucosinolates as glucobrassicin, 4-methoxy-glucobrassicin and 4-hidroxy-glucobrassicin, and phenolic acids and flavonoids as sinapic acid, 4-O-feruloyl quinic acid and quercetin-3-O-diglucoside was found. At day 6, the broccoli stalks reached the maximum counts in lactic acid bacteria (>8 log cfu g^{-1}), remaining stable until day 12 at 4 °C, including as the main genera *Lactobacillus* and *Leuconostoc*. Metagenomic analysis identified 1009 bacteria genera; *Latilactobacillus sakei* was the most predominant lactic acid bacteria species, followed by *Lactobacillus curvatus, Leuconostoc kimchi* and *Leuconostoc carnosum*. This is an example of a vegan and functional food product, based on a circular economy model, using broccoli by-products.

1. Introduction

The current trends in fermented-based vegetable foods are growing and this is likely to continue into the next decade. The value of these foods is in excess of $500 million in the U.S. market, with the global market being estimated at between five and 15 times more than the U.S. market (Schilling & Campbell, 2021). The oldest documents reporting on fermented foods date from 13,000 BC, primarily mediated by spontaneous fermentation by autochthonous microorganisms in the raw material. At that time, the main objectives were to extend their stability and their long-term storage at room temperatures. However, in recent years, numerous studies have shown the nutritional and health benefits brought about by the potentially probiotic effect of the microbiology that grows on this type of product, providing functional ingredients and improved digestibility. Lactic acid bacteria (LAB) are major microorganisms used for probiotic purposes which are usually employed in the food industry as starter or autochthonous cultures in the production of fermented foods such as yogurt, cheese, sausages, cabbage, cucumber, pepper, and other vegetable matrices. Genera of the LAB include *Lactobacillus-, Leuconostoc, Weisella, Enterococcus, Aerococcus,* and normally species such as *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum* and *Lactobacillus rhamnosus* commonly develop in vegetable-based fermented foods (Lahtinen, Ouwehand, Salminen, & von Wright, 2011). Moreover, the WHO reported the health benefits of consuming foods with a probiotic content in gastrointestinal infections and bowel disorders, reduction of serum cholesterol, an enhanced immune system, and a lower risk of colon cancer (FAO/WHO, 2001). Other metanalyses highlight the evidence of the health benefits of fermented foods in respiratory tract infections, gestational diabetes, and Crohn's disease (Limketkai, Akobeng, Gordon, & Adepoju, 2020).

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The fruit and vegetable industry generates vast amounts of bio-residues which present functional compounds like fibre, protein, peptides, vitamins, polyphenols, and glucosinolates with benefits in health and/or reductions in the progression of chronic diseases such as diabetes and obesity, and cardiovascular and intestinal disease (Gil-Martín et al., 2022). Strategies have been focused on reducing food loss, zero-waste, by-product revaluing to promote a circular economy, support the low-carbon economy, and improve efficiency in the food sector.

More than 25 million tonnes of cauliflower and broccoli are produced in the world (FAOSTAT, 2020). Broccoli (*Brassica oleracea* var. *italica*) is very rich in functional compounds including phenols, vitamin C and glucosinolates (Vallejo, Tomás-Barberán, & García-Vigueras, 2002). However, in broccoli, only 15% of the total biomass is useable, whilst the roots, stalk, and leaves are wasted, under-using about 17, 21, and 47%, respectively (Liu, Zhang, Ser, Cumming & Kum, 2018) with the consequent waste of limited resources such as water, land, and energy. Nevertheless, some authors approach broccoli by-products as part of ingredients in novel sauces and bakery products (Castillejo, Martínez-Hernández, & Artés-Hernández, 2021; Drabińska, Ciska, Szmatowicz, & Krupa-Kozak, 2018; Krupa-Kozak et al., 2021). However, in the case of the broccoli stalk, most of it is cut off in the industry, with the broccoli florets being the marketable part (Fig. 1). Therefore, the aim of this research was to revalue the broccoli stalk, designing a novel fermented food product with probiotic potential and which is rich in functional compounds. To this end, the microbiome profile, functional compounds (individual phenols and glucosinolates), and the sensory quality are evaluated in this new food product.

2. Materials and methods

2.1. Product processing

Broccoli stalks (*Brassica oleracea* L. *var Italica, cv. Parthenon*) were obtained from Levante Sur Coop. in the southeast of Spain (La Palma, Cartagena). The broccoli stalks were cut into 6 mm slices using an automatic slicer (Eurocort F250E, Spain) and jar packed adding brine (6% w/v) using commercial non-refined salt. Each glass jar contained broccoli stalk slices (80 g) and 120 mL of brine. For the fermentation process, an airlock was used in the top of the lids to avoid external air entering and thus to maintain the anaerobic conditions. Two dressings were also studied to achieve consumer acceptability and to increase functional compounds, namely with the use of garlic and mustard. Two garlic cloves (~8 g) were added (garlic dressing, GD) or 10 g of milled mustard seed (mustard dressing, MD) to the glass jars, with the brine and stalk slices. No dressing (ND) was used, only the salt, in the control treatment. The natural and spontaneous fermentation condition, with no inoculum added, was carried out at 25 °C in dark conditions for six days. After that period, the samples were stored under refrigerated conditions (4 °C) for a further six days. Every three days, samples were collected for microbiological, functional, and sensory evaluations. The experiment was conducted in triplicate, with three glass jars being used for each treatment (type of dressing and day of analysis).

2.2. Physico-chemical analysis

2.2.1. pH and total acidity (TA)

The pH during fermentation was measured using a pH meter (Crison 501, Barcelona, Spain) and TA was determined by the titration of the covering liquid (10 mL) using 0.1 M NaOH to 8.1 (T50, Metter Toledo, Milan, Italy) and expressed as g equivalent lactic acid per 100 mL of covering liquid.

2.2.2. Colour measurement

Colour was determined using a colorimeter (model CR-400, Minolta, Tokyo, Japan) calibrated with a white reference plate. Measurements of the slices of broccoli stalk were recorded using the standard tristimulus parameters. The L*, a*, b* colour values were determined using the CIELAB system, and hue angle (°h as $\tan^{-1}$ (b*/a*)) and chroma (C; $(a^{*2} + b^{*2})^{1/2}$) was calculated.

2.2.3. Firmness of slices

A puncture test was used to evaluate firmness of the slices, based on the resistance of each one to pressure applied by a texturometer (LFRA 1500 Texture Analyzer, BrookField, Engineering Laboratories, USA). During the test, a 4.5 mm diameter flat-head stainless-steel cylindrical probe penetrated the middle of the longitudinal axis of the slice (9 mm depth) at a speed of 6 mm s^{-1}. The results were expressed in N.

2.3. Microbial cultures and microbiological analysis

The microbiological analysis was performed following standard methods. To determine the microbial quality of fermentative products, 30 g of broccoli stalk slices were homogenised for 1.5 min in 270 mL of sterile peptone buffered water, with a 400 Lab Stomacher (Seward Medical, London, UK). Dilutions were made in peptone water, as needed, for plating. The enumeration of microbial groups was performed using the following media (Scharlau Chemie S.A., Barcelona, Spain) and culture conditions: Total lactic acid bacteria (LAB) were quantified by plating on Man, Rogosa and Sharpe agar (MRS), and anaerobic incubation (Thermo Scientific™ Oxoid AnaeroGen) at 30 °C for 72 h; mesophilic aerobic bacteria on plate count agar, incubated at 30 °C for 72 h; yeast and mould using rose bengal chloramphenicol agar and incubated at 25 °C for 96 h. All microbial counts were reported as log cfu/g (colony forming units per gram of sample).

Fig. 1. Broccoli with floret, leaves, and stalks (left). Stalks (by-products) from the industry (right).

2.4. Metatranscriptomic analysis of fermented broccoli stalk

The DNA was extracted from the fermented broccoli stalk at days 0, 3, 6, and 12 for each dressing treatment with the DNeasy PowerSoil Kit (Qiagen. Hilden, Germany) following the manufacturer's protocol: Metagenomics studies were performed by analysing the variable regions V3–V4 of the prokaryotic 16S rRNA gene sequences, which gives 460 bp amplicons in a two-round PCR protocol. In the first step, PCR was used to amplify a template out of a DNA sample using specific primers with overhang adapters attached to those flanks regions of interest. The full-length primer sequences to follow the protocol targeting this region were: Forward Primer: 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGAG and Reverse Primer: 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

In the second step and using a limited-cycle PCR, sequencing adapters and dual index barcodes, Nextera® XT DNA Index Kit, FC-131-1002 (Illumina, San Diego, CA, USA), were added to the amplicon for sequencing pooled together in the MiSeq sequencer with the MiSeq® Reagent Kit v2 (500 cycle) MS-102-2003. The 16S rRNA gene sequences obtained were analysed at the Basespace (Illumina) with the 16S Metagenomic App that performs taxonomic classification using a taxonomic database. The algorithm used is a high-performance implementation of the Ribosomal Database Project (RDP) Classifier described in Wang, Garrity, Tiedje, and Cole (2007).

2.5. Determination of bioactive compounds

2.5.1. Sample preparation

Ten broccoli stalk slices for each treatment, including the raw broccoli stalks at day 0, were immediately frozen in liquid nitrogen, milled, and stored at −80 °C until the analysis. The covering liquid (CL) was also stored at −80 °C and analysed. For the antioxidant capacities, total polyphenolic content (TPC), and the quantification and identification of individual phenolic acids and flavonoids, 2 g of the frozen sample in 15 mL of 70% methanol acidified with 1% of formic acid and sonicated was used. Samples were then filtered through four layers of cheesecloth and centrifuged at 3220×*g* for 10 min at 4 °C. The supernatant was used for TPC, antioxidant assays, and for the identification and quantification of phenolic acids and flavonoids by LC-MS/MS (previously filtered by 0.2 μm, nylon filter). The CL was also LC-MS/MS analysed with a centrifuged step to clarify the sample. For the quantification and identification of intact glucosinolates, 2 g of the frozen sample was used in 10 mL of 70% methanol, vortex and incubated for 10 min at 70 °C for myrosinase inactivation. Subsequently, the extract was filtered through four layers of cheesecloth and centrifuged at 3220×*g* for 10 min at 4 °C, followed by filtering prior to the LC-MS/MS analysis.

2.5.2. Total polyphenolic content and antioxidant capacity

The TPC and antioxidant capacities were measured using a multiscan plate reader (Tecan infinite M200, Männedorf, Switzerland); with TPC being determined in accordance with (Martínez-Sánchez, Guirao-Martínez, Martínez, Lozano-Pastor, & Aguayo, 2019) the antioxidant assays, ferric-reducing antioxidant power (FRAP) (Benzie & Strain., 1999) and Trolox Equivalent Antioxidant Capacity (TEAC) (Re et al., 1999).

2.5.3. Identification and quantification of phenolic compounds

Individual phenolic compounds content and their identification in the broccoli stalk slices and covering liquid were analysed in accordance with (Thomas, Badr, Desjardins Gosselin & Angers, 2018) with slight modifications. The Agilent 1200 liquid chromatography (Santa Clara, CA, USA) equipped with a G1311B quaternary pump, G1329B Standard Autosampler, G1316A column heater, and coupled to a 6420 triple-quadrupole mass spectrometer (QqQ) with an electrospray ionisation source (ESI) was used for this purpose. The separation of phenolic compounds was achieved on a reverse-phase column Luna Omega C18 (2.1 × 100 mm; 3 μm). The mobile phase consisted of A as 0.1% formic acid in water and B as 0.1% formic acid in acetonitrile. The analysis was performed in negative mode: the autosampler was injected with 5 μL of the sample, the oven temperature was set at 35 °C and the gradient was 0.1–2% B for 15 min, 2–31% B in 22 min, 31–95% B until 47 min of analysis with 5 min of isocratic conditions at 95% B. The capillary voltage, gas temperature, gas flow and nebuliser were set at 4000V, 350 °C, 11 L/min and 40 psi, respectively. Individual phenolic compounds were identified according to the four levels described by (Schymanski et al., 2014). Retention time, MS and MS/MS fragmentation spectra were compared with a commercial standard or tentatively identified comparing their fragmentation pattern with available bibliographic data and in the MassBank Europe, MassBank of North America and PubChem databases. Individual phenolic compounds were quantified by dynamic multiple reaction monitoring (dMRM) considering one transition for quantification and another for qualitative aspects at the specific instrumental conditions shown in Table S1. Chlorogenic acid derivatives were quantified as chlorogenic acid equivalent, rutin as flavonoids, sinapic acid as sinapic acid derivatives; with all of them being expressed as mg kg^{-1} per FW, and mg L^{-1} for the covering liquid.

2.5.4. Identification and quantification of glucosinolates

The analysis was performed in negative mode, with the same instrument, column, and mobile phases as in Section 2.5.3, and the conditions as per Capriotti et al. (2018). The autosampler was set to inject 5 μL of the sample that was eluted with a flow of 0.250 mL/min and the gradient was started 5–50% B (25 min), 50–95% B (5 min), 95–95% B (5 min), 95–5% B (10 min), at 40 °C. The mass analysis was operated in negative mode at these conditions: drying gas (N_2) temperature 350 °C, with a flow rate of 12 L/min, nebuliser gas pressure 30 psi, and capillary voltage 3000 V. Glucosinolates were identified following the conditions, classifications and strategies previously described in the analysis of individual phenolic compounds. Individual glucosinolates were quantified by dynamic multiple reaction monitoring (dMRM) considering one transition for quantification and another for qualitative aspects at the specific instrumental conditions shown in Table S1. Aliphatic glucosinolate was quantified as glucoraphanin equivalent and indolic glucosinolate as glucobrassicin equivalent; with all of them being expressed as mg kg^{-1} per fresh weight (FW), and mg L^{-1} for the covering liquid.

2.6. Sensory evaluation

The sensory evaluation was carried out by twelve panellists specially trained in fermented food, according to Dupas de Matos et al. (2019). Prior to performing the testing sessions, the panellists agreed on the attributes that best described sensory changes. The panellists evaluated the degree of satisfaction of different attributes such as "pickled" aroma and taste, texture, and global acceptance, using a 9-point hedonic scale, 1 – extremely poor, 5 – acceptable, and 9 – excellent, for each attribute. For sourness and saltiness, 1 – extremely disbalanced, 5 – acceptable, and 9 – excellently balanced. The samples were shown to the panellists (aged 24–60), in a room at 20 °C, on white dishes, with blind codes. Each panellist received the samples in random presentation order. Water was provided for palate cleansing, so the possible residual parts from the previous sample could be removed.

2.7. Statistical analysis

A completely randomised design was performed with three replicates per treatment, where each glass jar constituted one replicate. A two-way ANOVA ($P < 0.001$) was carried out to determine the effect of storage time (linked to fermentation process and cold stored) and type of dressings. Mean values were compared by LSD multiple-range test to identify significant differences among treatments and interactions between factors.

3. Results and discussion

3.1. Physicochemical parameters in broccoli stalk slices and covering liquid

The brine had an initial pH of 7.85 and TA <0.01 g/100 mL (Table 1). The changes in pH depended on storage time ($p < 0.001$), decreasing in all treatments on the third day of fermentation (4.73–4.96) followed by a second reduction on day 6 (4.20–4.30), without significant changes to the end of the experiment. During the fermentation process, LAB produced lactic and acetic acids that reduced the pH and increased the TA. On day 6, a statistically significant increase in TA was found, compared to day 0, for all the dressing treatments (<0.01 *versus* 0.51 g lactic 100 mL^{-1}). Mustard dressing obtained the highest TA, probably due to the richness in acid compounds present in mustard seeds.

The broccoli stalk slices retained their firmness throughout the experiment (range 4.71–5.35 N), showing only a significant slight decrease ($p < 0.01$), in the garlic dressing at day 12 (Table 1). In general, the lightness and °h of the broccoli stalk slices significantly decreased with storage time and type of dressing, with the typical green colour of raw stalk slices changing to yellowness (days 9 and 12). These changes were significantly more pronounced in the mustard treatments. Nevertheless, this minor degradation in the natural pigments, changing chlorophyll to pheophytin and derivatives due to the loss of the central magnesium ion, was accompanied by enzymatic oxidation in the fermentation process (Mashitoa et al., 2021). Chroma levels ranged between 16 and 22, with increases at some specific moments, although such changes were not relevant and did not affect the sensory quality (data not shown).

3.2. Microbiological and metatranscriptomic analysis during spontaneous fermentation of broccoli stalks

This fermentation process was carried out by utilising autochthonous phyllosphere of the raw broccoli stalk. The LAB counts (Fig. S1) were low in the raw stalk, following the normal microbiota of raw vegetables (2.0–4.0 log cfu g^{-1}) (di Cagno, Coda, de Angelis, & Gobbetti, 2013). After three days of fermentation, the LAB had increased significantly in all treatments, with similar results (8.04–8.50 log cfu g^{-1}), and the LAB level was stable in all treatments during cold storage. Mesophilic bacteria also increased following a similar LAB trend. On day 0, the mould count was 2.08 log cfu g^{-1} and decreased to indetectable levels (<1.7 log cfu g^{-1}) from day 3 to the end of cold storage, due to the drop in pH by lactic acid metabolism, or the production of secondary metabolites such as organic acids, carbon dioxide, bacteriocins and antibiotics that compromised its growth (Satora, Skotniczny, Strnad & Piechowicz, 2021). Otherwise, the yeast counts increased on day 3, with small changes depending on the treatments.

Genetic information of the microbiome obtained during the fermentation process and cold storage was identified using metagenomic sequencing on days 3, 6, and 12 (Table S2). These analyses were performed at Q30 quality score, which means 88.78% of the whole reads had a sequencing error of <0.1%. More than 95% of reads were classified to genera in all samples, but 25–30% were classified to the species (not included). Bacterial diversity was calculated according to the Shannon diversity index which increased after 3–12 days (1.22–1.37), with a slight decrease on day 12 in the garlic and mustard dressings, which dropped to 1.20 and 1.12, respectively. These results confirm the increase in bacterial diversity through the fermentation process; this concurs with other authors who worked with different fermented products, including brassica species (Liang et al., 2018).

Fig. 2A and B shows the distribution of phylum and genus level, respectively. *Proteobacteria* and *Firmicutes* were the dominant phyla in all the samples during the fermentation process. *Proteobacteria*'s abundance dropped drastically (45.40–50.80%) on the third day, 7.04–12.25% on the sixth day, and only slightly on the last day. That decrease corresponded with the growth of the *Firmicutes* phylum. We identified 1009 genera in all samples, which shows the ~90% sum of abundance (Fig. 2B). The *Proteobacteria* phylum (*Parvibaculum, Pleomorphobacterium, Pseudomonas, Psychrobacter and Erwinia* genus*)* was displaced principally by the *Lactobacillus, Leuconostoc and Lactococcus* genera. These three genera belong to the lactic acid bacteria group (LAB), which includes *Weisella, Aerococcus, Enterococcus, Pediococcus, Carnobacterium, Streptococcus, Oenococcus, Vagococcus* and *Tetragenococcus* (Lahtinen et al., 2011) that grow in fermented brassica-based foods such as kimchi, sauerkraut, sour cabbage, gundruk, and pao cai, in which many authors have described similar microbial changes (Liu, Zhang, Zhang, Xin, & Liao, 2021). On day 0, the LAB constituted only 6.73% of the broccoli stalk microbiome, up to 56.90–59.83% on day 3, and more than ~70% on days 6 and 12. The LAB genera that appeared in all samples, by order of abundance, were: *Lactobacillus*, *Leuconostoc*,

Table 1
Physico-chemical parameters of broccoli stalk slices or covering liquid at day 0 (raw) and during the fermentation process (days 3 and 6, at 25 °C) followed by cold storage (days 9 and 12, at 4 °C) with different dressing treatments (ND: No-dressing, GD: Garlic, MD: Mustard).

Treatments	Time (d)	pH	TA	Firmness (N)	Lightness (L*)	° h
ND	0	$7.85^{Aa} \pm 0.03^{z}$	$<0.01^{Ba}$	$4.86^{Aa} \pm 0.02$	$79.44^{Aa} \pm 0.2$	$105.51^{Aa} \pm 0.09$
	3	$4.80^{Ba} \pm 0.14$	$0.23^{ABa} \pm 0.01$	$4.93^{Aa} \pm 0.11$	$79.23^{Aa} \pm 0.44$	$104.45^{Ba} \pm 0.64$
	6	$4.20^{Ca} \pm 0.10$	$0.53^{Aa} \pm 0.02$	$5.12^{Aa} \pm 0.05$	$78.63^{Aa} \pm 0.77$	$97.35^{Ca} \pm 0.64$
	9	$4.08^{Ca} \pm 0.01$	$0.33^{ABa} \pm 0.01$	$5.15^{Aa} \pm 0.18$	$77.54^{Ba} \pm 0.48$	$99.83^{Ca} \pm 0.49$
	12	$4.31^{Ca} \pm 0.06$	$0.25^{ABa} \pm 0.05$	$5.16^{Aa} \pm 0.16$	$77.28^{Ba} \pm 1.18$	$97.99^{Ca} \pm 0.86$
GD	0	$7.85^{Aa} \pm 0.03$	$<0.01^{Ba}$	$4.86^{ABa} \pm 0.02$	$79.44^{Aa} \pm 0.2$	$105.51^{Aa} \pm 0.09$
	3	$4.73^{Ba} \pm 0.03$	$0.26^{ABa} \pm 0.02$	$5.31^{ABa} \pm 0.28$	$78.57^{Aa} \pm 0.84$	$102.88^{Ba} \pm 0.53$
	6	$4.29^{Ca} \pm 0.09$	$0.51^{Aa} \pm 0.04$	$5.09^{ABa} \pm 0.03$	$78.93^{Aa} \pm 1.59$	$98.87^{Ca} \pm 0.97$
	9	$4.12^{Ca} \pm 0.11$	$0.35^{ABa} \pm 0.00$	$5.35^{Aa} \pm 0.16$	$77.67^{Ba} \pm 1.42$	$98.78^{Ca} \pm 0.33$
	12	$4.10^{Ca} \pm 0.17$	$0.32^{ABa} \pm 0.02$	$4.71^{Ba} \pm 0.18$	$77.47^{Ba} \pm 1.16$	$97.14^{Ca} \pm 1.59$
MD	0	$7.85^{Aa} \pm 0.03$	$<0.01^{Ba}$	$4.86^{Aa} \pm 0.02$	$79.44^{Ab} \pm 0.2$	$105.51^{Ab} \pm 0.09$
	3	$4.96^{Ba} \pm 0.08$	$0.53^{Aa} \pm 0.03$	$5.31^{Aa} \pm 0.2$	$77.37^{Ab} \pm 0.66$	$101.31^{Bb} \pm 0.38$
	6	$4.30^{Ca} \pm 0.09$	$0.50^{Aa} \pm 0.01$	$5.18^{Aa} \pm 0.03$	$76.57^{Ab} \pm 4.31$	$96.97^{Cb} \pm 0.45$
	9	$4.21^{Ca} \pm 0.01$	$0.55^{Aa} \pm 0.01$	$5.35^{Aa} \pm 0.04$	$70.76^{Bb} \pm 0.65$	$97.05^{Cb} \pm 0.25$
	12	$4.21^{Ca} \pm 0.04$	$0.53^{Aa} \pm 0.00$	$4.89^{Aa} \pm 0.28$	$69.67^{Bb} \pm 2.27$	$96.56^{Cb} \pm 0.51$
Time (A)		(0.37)***	(0.19)***	(0.35)***	(1.21)**	(1.35)***
Type of dressing (B)		NS	(0.13)***	(0.24)***	(1.09)***	(0.54)**
A x B		NS	(0.43)***	(0.60)**	NS	NS

[z]Means (n = 3 ± SE). For firmness 5 replicates were used per glass jar (n = 15). Means followed by different letters in the same column for the same factor (capital letters for time and lower-case for type of dressing) are significantly different according to LSD (least statistical difference, in brackets). Asterisks indicate significant differences at **p ≤ 0.01; ***p ≤ 0.001. NS: not significant. TA: Total acidity (g lactic acid 100 mL^{-1}) in covering liquid.

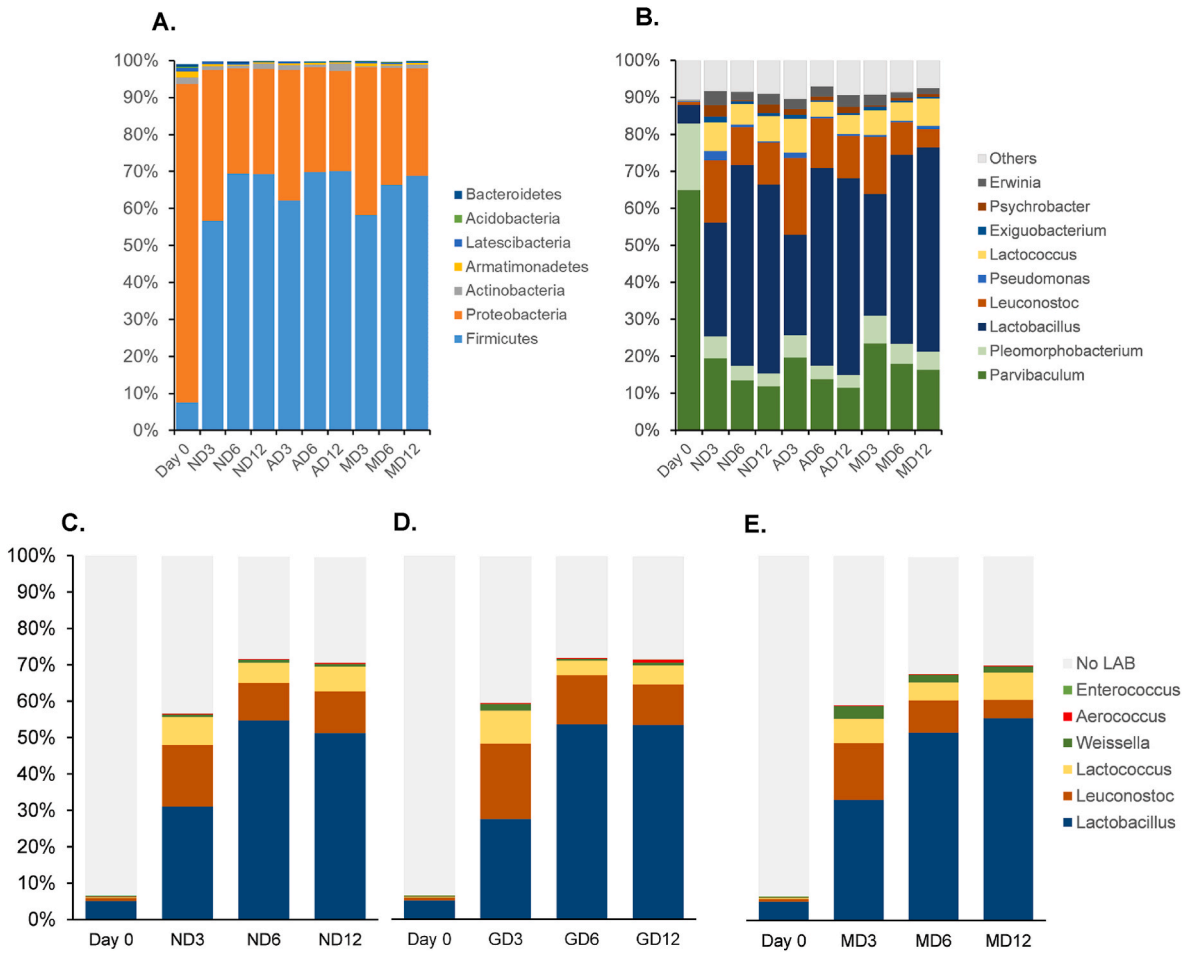

Fig. 2. Microbiological diversity distribution by phylum (A) and genus (B). Lactic acid bacteria genera of fermented broccoli stalk slices on day 0 (raw) and during the fermentation process (days 3 and 6, at 25 °C) followed by cold storage (day 12, at 4 °C) different dressing treatments (ND: No-dressing, GD: Garlic, MD: Mustard).

Lactococcus, *Weisella*, *Aerococcus* and *Enterococcus* (Fig. 2C, D and E). The *Lactobacillus* genus was the most abundant LAB throughout all the treatments, growing (22–28%) on day 3, 18–26% on day 6, and was stable during cold storage at the end of the experiment, with a slight increment in the mustard dressing (4%).

The heat map analysis (Fig. 3) shows the distribution and relationship of the 10 most abundant species and ordered by relative abundance. Approximately, 42.92–47.60% reads were classified to species taxonomic level, except the D0 sample with only 25.92%. The different colours were measured according to the Z-Score of species reads and applied hierarchical clustering to the row with Euclidean's method distance measurement. There were three principal groups: D0 treatment (raw broccoli stalk slices) formed the first group; treatments on day 3 (ND3, GD3 and MD3) constituted the second group; whilst the remaining samples on days 6 and 12 established the third group. No *Parvibaculum* species were identified, despite it being the most abundant in the D0 samples, followed by the *Pleomorphobacterium* genus. For that reason, we found *Pleomorphobacterium xiamenense* (2nd row; Fig. 3) as the most abundant in the D0 sample. The amount of that species dropped drastically after day 3 (18.0–5.9–7.5% in all treatments, and 3.5–4.8% maintained in next days). The principal bacteria identified in all samples was *Latilactobacillus sakei* subsp. *carnosus* (CCUG 31331) – basonym of *Lactobacillus* – a microaerophile, mesophilic, gram-positive bacterium that is normally isolated from fermented meat. Some authors have reported *L. sakei* as the predominant bacteria group in kimchi fermentation, and specifically subsp. *carnosus* (Jung et al., 2013). This species was evaluated as being protective and prolonging the shelf-life in cooked meat products due to a non-bacteriocinogenic mechanism, since the depletion of nutrients like vitamins, minerals, trace elements, peptides cause the antagonistic interaction phenomena or quorum sensing (Vermeiren, Devlieghere, & Debevere, 2006). In addition, *L. sakei* has health benefits that include a positive effect on metabolic disorders such as obesity and fatty liver (Won, Seo, Kwon, Park, & Yoon, 2021).

Other LAB species, with a relative abundance of 1–5% per species that grew in our fermented broccoli stalk slices with different progressions were *Lactobacillus curvatus* (NCFB 2739), *Leuconostoc kimchii* (IH25^T) and *Leuconostoc carnosum* (SML40). All of them have been reported in other food products and fermented brassicas (Maoloni et al., 2020). Generally, these three strains showed antimicrobial activities against pathological bacteria and moulds such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Aspergillus niger*. Other authors described health benefits and immunomodulatory activity in the mucosal system, as in ulcerative colitis (Jo et al., 2016).

3.3. Total phenolic compounds and antioxidant capacity

The TPC in raw broccoli stalk slices was 43.22 mg GAE/100 g (Table 2) and this concentration increased significantly in all treatments with the fermentation process, obtaining the maximum levels on day 3, for no dressing and garlic dressing treatments and on day 6, for mustard dressing. The TPC were influenced by the interaction of time and type of dressing ($p < 0.001$). Compared to no dressing and garlic treatments, broccoli stalks slices from the mustard dressing obtained the highest TPC levels, 2.5-fold more than the initial level (108.06–116.46 mg GAE/100 g). This increase in TPC measured in slices with the mustard dressing

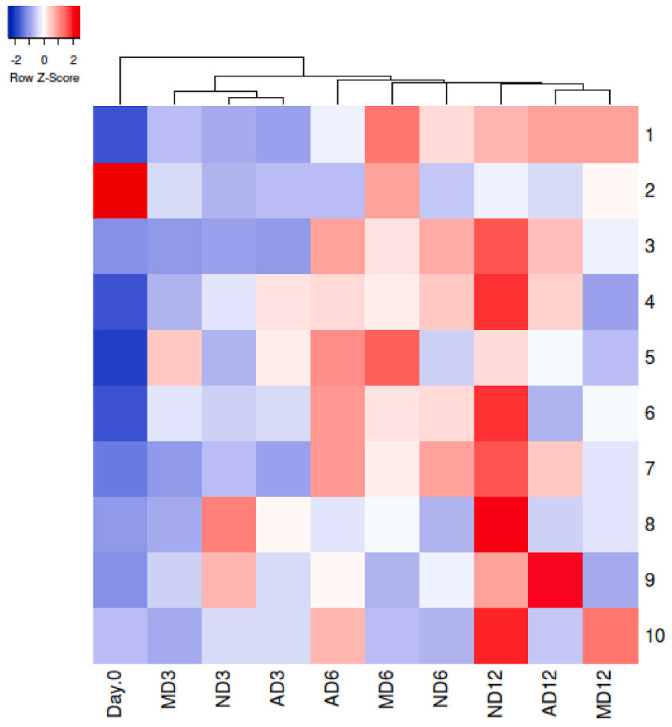

Fig. 3. Heatmap cluster of the top 10 at species level identified in the broccoli stalk slices on day 0 (raw) and during the fermentation process (days 3 and 6, at 25 °C) followed by cold storage (day 12, at 4 °C) with different dressing treatments (ND: No-dressing, GD: Garlic dressing, MD: Mustard dressing). Species identified and its 16S rRNA sequence: (1) *Lactobacillus sakei* (AY204889), (2) *Pleomorphobacterium xiamenense* (HQ709062), (3) *Lactobacillus curvatus* (AM113777), (4) *Leuconostoc kimchi* (AF173986), (5) *Leuconost carnosum* (A^B022925), (6) *Erwinia persicina* (U80205), (7) *Lactobacillus graminis* (AM113778), (8) *Psychrobacter alimentarius* (AY513645), (9) *Erwinia billingiae* (JN175337), (10) *Corynebacterium glutamicum* (AF314192).

came from the intrinsic polyphenol content in these seeds which transferred from the dressing to the slices (Nicácio, Rodrigues, Visentainer, & Maldaner, 2021). Regarding the antioxidant capacity in broccoli slices, FRAP increased from day 0–3, 0.25 to 0.52 and 0.95 mmol Fe^{+2}/100 g (Table 2). These increments were statistically significant ($p < 0.001$) in ABTS measurements, ranging from 113.05 to 189.51 and 215.25 mmol Trolox equivalents/100 g, with the significantly higher concentration in the mustard dressing treatment. The fermentation process of the broccoli stalk slices induced structural breakdown on the vegetable wall and could liberate and/or induce the synthesis of new functional compounds such as polyphenols, showing an increase in antioxidant activity (Hur, Lee, Kim, Choi, & Kim, 2014). From day 3, the antioxidant capacity, both FRAP and ABTS, was decreasing until the end of the experiment only being statistically significant in ABTS. As found in TPC, the mustard dressing provided the broccoli slices with significantly higher antioxidant levels (measured as ABTS).

Regarding the covering liquid (Table 2), the highest TPC concentration ($p < 0.001$) was obtained on day 6 (70.78 mg GAE/100 mL, average for the three dressings), decreasing during the cold storage (62.60 mg GAE/100 mL). The TPC contents were also influenced by the type of dressing ($p < 0.001$), with mustard dressing having the highest levels (132 mg GAE/100 mL), followed by garlic (24.35 mg GAE/100 mL) and no dressing (21.35 mg GAE/100 mL). For antioxidant capacity, the interaction between storage time and the type of dressing, showed the mustard dressing had the highest antioxidant capacity (FRAP and ABTS), and day 6 (end of fermentation) was the moment with maximum antioxidant levels in the dressing; they then slowly declined with cold storage (this trend was only significant for ABTS measurements). The fact that the highest concentration of TPC and antioxidant compound in the broccoli stalks slices was found on day 3, but in the covering liquid on day 6, would explain the diffusion of these compounds from the broccoli slices to the covering liquid due to the increase in cell wall permeability by the fermentation process and its induction to structural breakdown on the plant wall (Hur et al., 2014). Therefore, the broccoli stalk slices showed a reduction in functional compounds from day 3, whilst an increase was obtained in the covering liquid. In addition, the compounds present in the dressing as garlic and mustard seeds also helped to increase the functional compounds in the covering liquid. This

Table 2
Total phenolic compounds (TPC) and antioxidant capacity (FRAP and ABTS) of broccoli stalk slices and covering liquid on day 0 (raw) and during the fermentation process (days 3 and 6, at 25 °C) followed by cold storage (days 9 and 12, at 4 °C) with different dressing treatments (ND: No-dressing, GD: Garlic, MD: Mustard).

Treatments	Day	TPC		FRAP		ABTS	
		Broccoli stalk	Covering liquid	Broccoli stalk	Covering liquid	Broccoli stalk	Covering liquid
ND	0	$43.22^{Ba} \pm 2.02^{z}$	–	$0.25^{Aa} \pm 0.01$	–	$113.05^{Ba} \pm 1.51$	–
	3	$50.35^{Ab} \pm 2.98$	$5.08^{Bb} \pm 1.08$	$0.55^{Aa} \pm 0.07$	$0.03^{Ab} \pm 0.01$	$194.27^{Ab} \pm 14.99$	$16.94^{Cb} \pm 0.94$
	6	$20.52^{Dc} \pm 4.84$	$29.19^{Ab} \pm 0.23$	$0.20^{Aa} \pm 0.04$	$0.28^{Ab} \pm 0.01$	$79.42^{Cb} \pm 9.68$	$137.74^{Ab} \pm 12.00$
	9	$24.95^{Cb} \pm 1.52$	$28.73^{Ab} \pm 1.08$	$0.17^{Aa} \pm 0.03$	$0.24^{Ab} \pm 0.02$	$76.85^{Cb} \pm 12.36$	$103.22^{Bc} \pm 1.42$
	12	$25.55^{Cb} \pm 1.73$	$14.15^{Ab} \pm 0.03$	$0.07^{Ab} \pm 0.01$	$0.20^{Ab} \pm 0.03$	$41.34^{Dc} \pm 2.85$	$97.99^{Bc} \pm 4.82$
GD	0	$43.22^{Aa} \pm 2.02$	–	$0.25^{Aa} \pm 0.01$	–	$113.05^{Ba} \pm 1.51$	–
	3	$47.08^{Ab} \pm 2.81$	$6.30^{Bb} \pm 0.96$	$0.52^{Aa} \pm 0.06$	$0.04^{Ab} \pm 0.01$	$189.51^{Ab} \pm 9.8$	$20.70^{Db} \pm 2.33$
	6	$28.71^{Bb} \pm 1.41$	$36.67^{Ab} \pm 2.05$	$0.16^{Aa} \pm 0.01$	$0.33^{Ab} \pm 0.04$	$53.55^{Cc} \pm 2.15$	$159.63^{Ab} \pm 28.01$
	9	$27.28^{Bb} \pm 5.25$	$24.59^{Ab} \pm 1.20$	$0.12^{Aa} \pm 0.02$	$0.22^{Ab} \pm 0.01$	$47.58^{Cc} \pm 7.39$	$94.97^{Cc} \pm 5.70$
	12	$23.06^{Cb} \pm 2.59$	$29.82^{Ab} \pm 0.82$	$0.21^{Aa} \pm 0.001$	$0.27^{Ab} \pm 0.03$	$50.12^{Cb} \pm 6.67$	$123.11^{Bb} \pm 6.33$
MD	0	$43.22^{Ca} \pm 2.02$	–	$0.25^{Ba} \pm 0.01$	–	$113.05^{Ca} \pm 1.51$	–
	3	$110.76^{Ba} \pm 2.33$	$104.35^{Ba} \pm 12.41$	$0.95^{Aa} \pm 0.02$	$0.63^{Aa} \pm 0.06$	$215.25^{Aa} \pm 2.67$	$366.49^{ABa} \pm 24.78$
	6	$116.46^{Aa} \pm 2.28$	$146.47^{Aa} \pm 8.10$	$0.63^{Aa} \pm 0.02$	$1.00^{Aa} \pm 0.06$	$173.84^{Ba} \pm 5.13$	$376.20^{Aa} \pm 19.85$
	9	$108.06^{Ba} \pm 0.55$	$141.56^{Aa} \pm 5.38$	$0.58^{Aa} \pm 0.03$	$0.83^{Aa} \pm 0.01$	$171.87^{Ba} \pm 4.78$	$356.35^{Ba} \pm 17.59$
	12	$115.41^{Aa} \pm 2.32$	$135.60^{Aa} \pm 3.38$	$0.59^{Aa} \pm 0.02$	$0.77^{Aa} \pm 0.01$	$172.33^{Ba} \pm 1.86$	$309.45^{Ca} \pm 2.83$
Time (A)		(2.15)***	(2.72)***	(0.22)***	(0.21)***	(3.15)***	(4.67)***
Dressing (B)		(1.47)***	(2.53)***	(0.15)***	(0.19)***	(2.15)***	(4.35)***
A x B		(4.90)***	NS	(0.50)***	(0.38)**	(7.19)***	(11.45)***

[z]Means ($n = 3 \pm$ SE). Means followed by different letters in the same column for the same factor (capital letters for time and lower-case for type of dressing) are significantly different ($p \leq 0.05$) according to LSD (least statistical difference, in brackets). Asterisks indicate significant differences at $p \leq 0.001$. NS: not significant. TPC: mg GAE per 100 g for broccoli slices or 100 mL for covering liquid. FRAP: mmol Fe^{+2} per 100 g for broccoli slices or 100 mL for covering liquid. ABTS: Trolox equivalent antioxidant capacity: mmol TE per 100 g for broccoli slices or 100 mL for covering liquid.

suggests that this novel food product, both the slices as well as the covering liquid, should be ingested in order to benefit from the functional potential of the whole product.

3.4. Individual phenolic compounds changes during fermentation

In the supplementary material, the retention time, MS and MS/MS fragmentation data and mass spectrometer conditions for the glucosinolates and phenolic compounds identified are listed (Table S1). Eleven phenolic compounds, principally hydroxycinnamic acids and flavonoids, were identified in our raw stalk samples. These were classified into caffeic acid derivatives (chlorogenic acid, rosmarinic acid), sinapic acid derivatives (sinapic acid, sinapic acid hexose, 1-sinapoyl-2-feruloyl-gentibiose, 1,2-disinapoyl-gentiobiose), ferulic acid derivatives (4-O-feruloyl quinic acid), and flavonoids (kaempferol-3-O-diglucoside-7-O-glucoside, quercetin-3-O-diglucoside-7-O-glucoside, kaempferol 3-O-diglucoside, quercetin-3-O-diglucoside), all of which were characterised in broccoli stalks (Thomas, Badr, Desjardins, Gosselin, & Angers, 2018). The trend of individual phenolics (Table 3) content was similar to the TPC assay (Table 2) where a slight decrease in no dressing and garlic dressing treatments but a high peak increment in the broccoli stalk slices with the mustard dressing was shown. On day 0 in the raw broccoli slices (Fig. S2), the total individual phenolics analysed by mass spectrometry was 3.66 ± 0.37 mg/kg, with the most abundant phenolic acids identified being 4-feruloyl quinic acid, sinapic hexose and chlorogenic acid, and flavonoids such as quercetin-3-O-diglucoside-7-O-glucoside and kaempferol-3-O-diglucoside-7-O-glucoside. During the experiment, a rapid reduction of multi-glycosided flavonoids (quercetin-3-O-diglucoside-7-O-glucoside and kaempferol-3-O-diglucoside-7-O-glucoside) to trace levels was found, but this was related to an increase in diglycosided phenolics as quercetin-3-O-diglucoside and kaempferol-3-O-diglucoside. In the same way, the reduction of sinapoyl hexose, 1-sinapoyl-2-feruloyl--gentibiose and disinapoyl gentibiose contents were related with the increase in sinapic acid. As Septembre-Malaterre, Remize, and Poucheret (2018) explained, these results suggest the conversion by esterase enzymatic activity from the LAB, which catalyses the hydrolysis of ester groups, improving the bioavailability of phenolic acids and flavonoids. Moreover, LAB may degrade these phenolic compounds by the activities of phenolic acid decarboxylase enzymes, which explains why some individual phenolics decreased during the fermentation process e.g., chlorogenic acid, rosmarinic acid, and feruloyl quinic acid as Septembre-Malaterre et al. (2018) also reported. Phenolic compounds of the broccoli stalk slices with the mustard dressing increased from 3.66 at day 0–52.85–59.54 mg/kg with the fermentation process, mainly sinapic acid which ranged from 42.81 to 52.70 mg/kg during the experiment. However, total individual phenolics from no dressing or garlic dressing decreased throughout the fermentation (3.66 mg/kg at day 0–1.09, and 1.30 mg/kg, respectively). The addition of milled mustard seeds in the dressing explains the rise in individual phenolic compounds due to the high sinapic acid content in mustard (Nicácio et al., 2021) that is released from the seeds and transferred to the broccoli stalk slices.

Concerning the covering liquid, on day 3 a diffusion of phenolic compounds from the broccoli slices to the covering liquid was found (Table 4). For example, 4-feruloyl quinic acid increased in all treatments on day 6 (0.52–0.70 mg/L), with a slight decrease in the garlic and mustard dressing but with a greater reduction in the no dressing and kept those levels until day 12. At the end of the experiment, the garlic dressing stood out for its increase in quercetin-3-O-diglucoside, whilst in the rest of the treatments it decreased. Conversely, in the broccoli stalk slices with the mustard dressing, the covering liquid of that treatment also rose in sinapic acid content, we quantified 71.52 mg/L on day 3 but this was falling during the experiment. Quercetin-3-O-diglucoside-7-O-glucoside, kaempferol-3-diglucoside-7-O-glucoside and rosmarinic acid were not detected in any covering liquid because of their low concentrations in the broccoli slices.

Numerous clinical trials have justified the health bioactivity of daily broccoli intake, regarding topics such as cancer, oxidative stress and inflammation (Marino et al., 2021). López-Chillón et al. (2019) showed a decrease in chronic inflammation in overweight subjects with a dietary serving of 120 mg of glucosinolates daily. Moreover, other bioactive compounds such as sinapic acid and chlorogenic acid demonstrated antioxidant, anti-cancer, -diabetic, -hypertensive, -bacteria and neuroprotective properties, among others (Pandi & Kalappan, 2021). In addition, the increase in functional broccoli compounds in the covering liquid suggests a diffusion from the broccoli stalk to the liquid, which also offers functional potential. The optimal moment for consumption, when functional compounds are at their highest, should be around day 3, when the fermentation process has just finished.

3.5. Individual glucosinolates changes during fermentation

Ten glucosinolates were identified in the raw stalk (Fig. S3): six belong to aliphatic glucosinolates (glucoiberin, glucoraphanin, glucoiberverin, glucotropaeolin, glucoerucin and gluconasturtiin) and four belong to indolic glucosinolates (4-hidroxy-glucobrassicin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin). All of them had been previously found in broccoli and brassica species (references cited in Table S1). Other glucosinolates such as progoitrin, glucoalyssin, and gluconapin were reported in broccoli florets but were absent in our stalk slices, due to the low or no concentration of these compounds in other plant parts such as the stalks or leaves (Thomas et al., 2018).

The content of individual glucosinolates and phenolic compounds and the changes during the fermentation process in the broccoli stalk slices and covering liquid are shown in Tables 3 and 4 Initially, the broccoli stalk had 73.42 ± 2.11 mg/kg of total glucosinolates, of which 60–70% were indolic (mainly glucobrassicin, neoglucobrassicin and 4-methoxyglucobrassicin) and the rest were aliphatic, principally glucoerucin content that showed approximately 22% of total glucosinolates, followed by glucoraphanin and gluconasturtiin (2.49 and 2.33%, respectively).

These results are in accordance with those of other authors who analysed broccoli stalks and reported a high content in indolic and glucoerucin glucosinolates (Thomas et al., 2018), although the glucoraphanin content was higher than in our results. During the fermentation process, the evolution of individual glucosinolates in all treatments showed a similar trend, with an increment on day 3, ranging from 96.29 to 112.11 mg/kg, due to a rise in the concentration in glucoerucin, gluconasturtiin, and neoglucobrassicin. Glucobrassicin remained stable and the rest of the glucosinolates decreased. On day 6, glucosinolates had dropped to between 87 and 96% in all treatments, to 10.62–13.60 mg/kg in no dressing and garlic dressing and 4.61 mg/kg in mustard dressing. On day 9, glucosinolates decreased with slight differences except in the mustard treatments, which showed a significant decrease with the absence of glucoiberin and glucoraphanin, and glucoerucin and glucobrassicin were not detected at the end of the experiment. On day 12, 4-methoxyglucobrassicin, neoglucobrassicin, glucoerucin and glucobrassicin were the most stable and dominant glucosinolates in no dressing and garlic dressing. This trend had been previously reported in other studies using fermentation processes in brassica species (Sarvan et al., 2013), suggesting that glucosinolates decreased due to the disruption of plant tissue by the cut and/or microbiological enzymatic activity released the endogenous myrosinase. The hydrolysis of glucosinolates released sulphate, D-glucose, and sulphur- or nitrogen-compound as isothiocyanates, thiocyanates, nitriles, and thiones, due to the breakdown of the glucosinolate-thioglucoside bond by myrosinase and, in an acidic environment, mainly isothiocyanates are formed (Szutowska et al., 2021). Ciska, Honke, and Drabińska (2021) reported a decrease in glucosinolates in sauerkraut and its juice products, but an increase in ascorbigen and isothiocyanates during storage time (13 weeks), which exhibited anti-cancer properties. According to those results, our broccoli stalk slices should be rich in isothiocyanates providing a functional food with anti-cancer potential.

Table 3
Individual polyphenol compounds and intact glucosinolate (GLS) (mg/kg FW) in broccoli stalk slices on day 0 (raw) and during the fermentation process (days 3 and 6, at 25 °C) followed by cold storage (days 9 and 12, at 4 °C) with different dressing treatments (ND: No-dressing, GD: Garlic, MD: Mustard).

	Day 0 (raw)	No-dressing				Garlic dressing				Mustard dressing			
Day		3	6	9	12	3	6	9	12	3	6	9	12
CHL	0.45 ± 0.18[z]	0.70 ± 0.04	0.27 ± 0.08	0.35 ± 0.02	0.26 ± 0.08	0.84 ± 0.07	0.20 ± 0.05	0.26 ± 0.09	0.36 ± 0.02	0.85 ± 0.08	0.34 ± 0.03	0.32 ± 0.01	0.30 ± 0.02
Q3dG7G	0.29 ± 0.02	tr	n.d.	n.d.	n.d.	tr	n.d.	n.d.	n.d.	tr	n.d.	n.d.	n.d.
K3dG7G	0.14 ± 0.00	tr	n.d.	n.d.	n.d.	tr	n.d.	n.d.	n.d.	tr	n.d.	n.d.	n.d.
SNP-Hex	0.90 ± 0.06	0.67 ± 0.11	0.06 ± 0.03	0.01 ± 0.00	0.01 ± 0.01	0.74 ± 0.11	0.01 ± 0.00	0.04 ± 0.02	0.01 ± 0.00	15.30 ± 1.83	0.88 ± 0.02	0.49 ± 0.11	0.28 ± 0.04
4FQa	0.99 ± 0.05	0.34 ± 0.02	0.16 ± 0.03	0.20 ± 0.02	0.17 ± 0.05	0.32 ± 0.04	0.15 ± 0.03	0.14 ± 0.02	0.18 ± 0.01	0.49 ± 0.05	0.25 ± 0.02	0.24 ± 0.02	0.25 ± 0.02
QdG	0.17 ± 0.01	0.18 ± 0.07	0.20 ± 0.09	0.47 ± 0.20	0.12 ± 0.04	0.17 ± 0.06	0.46 ± 0.11	0.35 ± 0.23	0.74 ± 0.05	0.30 ± 0.16	0.23 ± 0.10	0.36 ± 0.05	0.19 ± 0.10
SNP	0.38 ± 0.19	0.16 ± 0.01	0.16 ± 0.05	0.07 ± 0.01	0.70 ± 0.43	0.20 ± 0.03	0.04 ± 0.01	0.55 ± 0.20	0.11 ± 0.03	42.81 ± 8.22	51.46 ± 6.19	52.70 ± 4.99	52.01 ± 3.29
KdG	0.05 ± 0.00	0.06 ± 0.02	0.07 ± 0.03	0.11 ± 0.04	0.04 ± 0.01	0.07 ± 0.02	0.14 ± 0.02	0.09 ± 0.06	0.17 ± 0.02	0.07 ± 0.03	0.05 ± 0.02	0.09 ± 0.01	0.06 ± 0.03
RosA	0.09 ± 0.01	1.82 ± 0.38	0.19 ± 0.03	0.05 ± 0.00	0.02 ± 0.00	0.30 ± 0.04	0.11 ± 0.00	0.05 ± 0.01	0.02 ± 0.00	0.21 ± 0.03	0.12 ± 0.01	0.04 ± 0.00	0.03 ± 0.00
dSNPGb	0.25 ± 0.02	0.18 ± 0.04	0.01 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.15 ± 0.04	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.18 ± 0.02	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
SnFrGb	0.41 ± 0.03	0.24 ± 0.07	0.02 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.16 ± 0.05	0.02 ± 0.00	0.02 ± 0.01	0.04 ± 0.00	0.17 ± 0.02	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
ΣPhenols	**3.66 ± 0.37**	**3.64 ± 0.21**	**0.86 ± 0.10**	**0.95 ± 0.25**	**1.09 ± 0.48**	**2.11 ± 0.20**	**0.93 ± 0.14**	**1.27 ± 0.51**	**1.30 ± 0.05**	**59.54 ± 8.68**	**53.06 ± 6.16**	**53.98 ± 5.05**	**52.85 ± 3.21**
GIB	0.09[z]±0.00	0.04 ± 0.00	0.01 ± 0.00	tr	n.d.	0.04 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.04 ± 0.00	n.d.	n.d.	n.d.
GRP	1.83 ± 0.00	0.69 ± 0.00	0.28 ± 0.01	0.20 ± 0.01	0.16 ± 0.02	0.56 ± 0.06	0.31 ± 0.01	0.25 ± 0.06	0.25 ± 0.01	0.60 ± 0.03	0.03 ± 0.02	n.d.	n.d.
GIV	0.15 ± 0.01	0.10 ± 0.02	tr	tr	tr	0.16 ± 0.05	tr	0.02 ± 0.01	0.01 ± 0.00	0.43 ± 0.03	0.06 ± 0.01	0.07 ± 0.00	0.07 ± 0.01
4OH-GBS	3.32 ± 0.40	1.86 ± 0.23	0.09 ± 0.03	0.08 ± 0.01	0.05 ± 0.02	1.82 ± 0.42	0.05 ± 0.01	0.08 ± 0.04	0.06 ± 0.00	2.34 ± 0.29	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
GTP	1.94 ± 0.10	1.22 ± 0.13	0.57 ± 0.03	0.54 ± 0.08	0.49 ± 0.02	1.10 ± 0.03	0.58 ± 0.02	0.69 ± 0.10	0.54 ± 0.04	1.12 ± 0.12	0.53 ± 0.04	0.53 ± 0.02	0.51 ± 0.04
GER	16.75 ± 0.25	47.51 ± 1.50	4.70 ± 0.92	5.34 ± 0.54	3.95 ± 0.67	40.39 ± 3.64	2.53 ± 0.08	4.39 ± 1.00	5.27 ± 0.18	49.98 ± 2.41	0.42 ± 0.26	0.05 ± 0.02	n.d.
GBS	11.36 ± 0.28	11.21 ± 0.54	1.39 ± 0.15	1.01 ± 0.08	0.87 ± 0.20	9.64 ± 1.28	1.15 ± 0.19	1.23 ± 0.43	1.05 ± 0.08	12.15 ± 1.96	0.18 ± 0.11	0.01 ± 0.00	n.d.
NAS	1.71 ± 0.00	6.05 ± 1.07	0.60 ± 0.06	0.50 ± 0.07	0.45 ± 0.02	4.58 ± 0.86	0.52 ± 0.09	0.56 ± 0.01	0.53 ± 0.04	5.52 ± 1.22	0.07 ± 0.04	0.01 ± 0.00	0.01 ± 0.00
4 ME-GBS	19.61 ± 1.08	10.78 ± 0.81	2.46 ± 0.25	2.34 ± 0.30	2.09 ± 0.28	10.79 ± 1.81	2.12 ± 0.07	2.24 ± 0.44	2.08 ± 0.07	11.91 ± 0.55	2.82 ± 0.06	1.95 ± 0.19	1.57 ± 0.14
Neo-GBS	16.66 ± 0.02	25.46 ± 0.56	3.50 ± 0.12	2.80 ± 0.26	3.09 ± 0.60	27.23 ± 5.73	3.35 ± 0.49	3.07 ± 0.71	2.60 ± 0.13	28.01 ± 1.14	0.50 ± 0.31	0.03 ± 0.01	0.01 ± 0.00
ΣGLS	**73.42 ± 2.11**	**104.91 ± 1.41**	**13.60 ± 1.13**	**12.81 ± 1.14**	**11.15 ± 1.79**	**96.29 ± 10.98**	**10.62 ± 0.77**	**12.54 ± 1.90**	**12.39 ± 0.10**	**112.11 ± 2.77**	**4.61 ± 0.82**	**2.65 ± 0.23**	**2.21 ± 0.17**

[z]Mean (n = 3 ± SE). **Phenolic compounds:** Chlorogenic acid (CHL), quercetin-3-O-diglucoside-7-O-glucoside (Q3dG7G), kaempferol-3-O-diglucoside-7-O-glucoside (K3dG7G), sinapoyl hexose (SNP-Hex), 4-O-feruloyl quinic acid (4FQa), quercetin-3-O-diglucoside (QdG), sinapic acid (SNP), kaempferol 3-O-diglucoside (KdG), rosmarinic acid (RosA), 1,2-disinapoyl-gentiobiose (dSNPGb) and 1-sinapoyl-2-feruloyl-gentibiose (SnFrGb). **GLS (glucosinolates):** Glucoiberin (GIB), glucorafanin (GRP), glucoiberverin (GIV), 4-hydroxy-glucobrassicin (4OH-GBS), glucotropaeolin (GTP), glucoerucin (GER), glucobrassicin (GBS), gluconasturiin (NAS), 4-methoxy-glucobrassicin (4ME-GBS), neoglucobrassicin (Neo-GBS). n.d.: not detected. tr.: trace <0.01 mg/kg.

Table 4
Individual polyphenol compounds and intact glucosinolate (GLS) in covering liquid (mg/L FW) on day 0 (raw) and during the fermentation process (days 3 and 6, at 25 °C) followed by cold storage (days 9 and 12, at 4 °C) with different dressing treatments (ND: No-dressing, GD: Garlic, MD: Mustard).

	No-dressing				Garlic dressing				Mustard dressing			
Day	3	6	9	12	3	6	9	12	3	6	9	12
CHL	0.01 ± 0.01[z]	0.29 ± 0.01	0.59 ± 0.10	0.03 ± 0.01	0.01 ± 0.00	0.61 ± 0.33	0.48 ± 0.07	0.70 ± 0.03	0.08 ± 0.00	0.460 ± 0.09	0.35 ± 0.02	0.20 ± 0.02
Q3dG7G	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
K3dG7G	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SNP-Hex	0.04 ± 0.01	0.04 ± 0.00	0.05 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.06 ± 0.01	0.08 ± 0.01	0.06 ± 0.00	0.29 ± 0.01	1.22 ± 0.03	1.00 ± 0.05	0.54 ± 0.04
4FQa	0.11 ± 0.04	0.52 ± .02	0.45 ± 0.08	0.17 ± 0.06	0.11 ± 0.03	0.59 ± 0.12	0.37 ± 0.05	0.41 ± 0.02	0.24 ± 0.01	0.70 ± 0.04	0.74 ± 0.04	0.60 ± 0.02
QdG	0.04 ± 0.00	0.03 ± 0.00	0.23 ± 0.11	n.d.	0.04 ± 0.02	0.37 ± 0.23	0.17 ± 0.09	0.55 ± 0.03	0.20 ± 0.01	0.17 ± 0.06	0.09 ± 0.00	0.01 ± 0.01
SNP	0.04 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.04 ± 0.00	0.04 ± 0.01	0.08 ± 0.01	0.07 ± 0.00	0.07 ± 0.00	71.52 ± 2.91	31.41 ± 5.15	11.62 ± 0.58	16.31 ± 3.78
KdG	0.02 ± 0.00	0.02 ± 0.00	0.06 ± 0.03	tr	0.02 ± 0.01	0.11 ± 0.05	0.05 ± 0.03	0.12 ± 0.01	0.05 ± 0.00	0.04 ± 0.02	0.02 ± 0.00	tr
RosA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
dSNPGb	n.d.	n.d.	tr	n.d.	n.d.	tr	0.01 ± 0.00	tr	n.d.	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
SnFrGb	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
ΣPhenols	**0.29 ± 0.05**	**0.97 ± 0.04**	**1.49 ± 0.32**	**0.31 ± 0.06**	**0.29 ± 0.05**	**1.86 ± 0.68**	**1.28 ± 0.15**	**1.94 ± 0.09**	**72.41 ± 2.95**	**34.15 ± 5.30**	**13.87 ± 0.69**	**17.71 ± 3.85**
GIB	n.d.	tr	tr	tr	n.d.	tr	0.01 ± 0.00	0.01 ± 0.00	n.d.	n.d.	n.d.	n.d.
GRP	n.d.	0.12[z]±0.03	0.14 ± 0.02	0.16 ± 0.02	0.01 ± 0.01	0.15 ± 0.04	0.27 ± 0.01	0.16 ± 0.01	n.d.	n.d.	n.d.	n.d.
GIV	n.d.	0.004 ± 0.001	0.01 ± 0.00	0.01 ± 0.00	n.d.	0.01 ± 0.00	0.01 ± 0.00	tr	0.11 ± 0.01	0.08 ± 0.00	0.07 ± 0.00	0.05 ± 0.00
4OH-GBS	0.03 ± 0.02	0.33 ± 0.16	0.31 ± 0.02	0.17 ± 0.09	0.07 ± 0.03	0.35 ± 0.25	0.57 ± 0.18	0.33 ± 0.02	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
GTP	0.12 ± 0.01	0.55 ± 0.04	0.53 ± 0.12	0.49 ± 0.02	0.10 ± 0.02	0.62 ± 0.02	0.58 ± 0.08	0.51 ± 0.03	0.19 ± 0.01	0.53 ± 0.07	0.60 ± 0.04	0.50 ± 0.05
GER	0.07 ± 0.02	5.72 ± 1.76	7.36 ± 0.96	7.17 ± 0.26	0.16 ± 0.07	8.25 ± 3.27	8.90 ± 0.09	8.81 ± 0.08	0.01 ± 0.00	0.01 ± 0.000	tr	tr
GBS	0.05 ± 0.02	1.15 ± 0.44	1.22 ± 0.21	1.18 ± 0.25	0.10 ± 0.05	1.42 ± 0.66	1.89 ± 0.17	1.48 ± 0.10	n.d.	n.d.	n.d.	n.d.
NAS	tr	0.31 ± 0.10	0.51 ± 0.08	0.57 ± 0.04	0.01 ± 0.01	0.55 ± 0.26	0.66 ± 0.09	0.67 ± 0.06	tr	0.01 ± 0.00	tr	tr
4 ME-GBS	0.70 ± 0.02	5.17 ± 0.75	5.22 ± 0.92	4.55 ± 0.72	0.73 ± 0.26	5.77 ± 1.11	5.32 ± 0.16	6.09 ± 0.23	0.57 ± 0.10	2.43 ± 0.20	2.38 ± 0.15	1.53 ± 0.10
Neo-GBS	0.06 ± 0.02	2.22 ± 0.69	3.06 ± 0.47	4.04 ± 0.69	0.13 ± 0.07	3.70 ± 1.72	4.21 ± 0.15	3.41 ± 0.22	n.d.	n.d.	n.d.	n.d.
ΣGLS	**1.08 ± 0.05**	**15.65 ± 3.88**	**18.42 ± 2.65**	**18.41 ± 2.01**	**1.35 ± 0.49**	**20.88 ± 7.18**	**22.47 ± 0.03**	**21.55 ± 0.44**	**0.91 ± 0.10**	**3.08 ± 0.27**	**3.09 ± 0.15**	**2.12 ± 0.11**

[z]Mean (n = 3 ± SE). **Phenolic compounds:** Chlorogenic acid (CHL), quercetin-3-O-diglucoside-7-O-glucoside (Q3dG7G), kaempferol-3-O-diglucoside-7-O-glucoside (K3dG7G), sinapoyl hexose (SNP-Hex), 4-O-feruloyl quinic acid (4FQa), quercetin-3-O-diglucoside (QdG), sinapic acid (SNP), kaempferol 3-O-diglucoside (KdG), rosmarinic acid (RosA), 1.2-disinapoyl-gentiobiose (dSNPGb) and 1-sinapoyl-2-feruloyl-gentibiose (SnFrGb). **GLS (glucosinolates):** Glucoiberin (GIB), glucorafanin (GRP), glucoiberverin (GIV), 4-hydroxy-glucobrassicin (4OH-GBS), glucotropaeolin (GTP), glucoerucin (GER), glucobrassicin (GBS), gluconasturiin (NAS), 4-methoxy-glucobrassicin (4ME-GBS), neoglucobrassicin (Neo-GBS). n.d.: not detected. tr.: trace <0.01 mg/kg.

Concerning the covering liquid, as expected, we did not detect glucosinolates on day 0 (data not shown). However, on day 3, the total glucosinolates content ranged from 0.91 to 1.35 mg/L and resulted in a rapid increase at day 6 (end of fermentation), remaining stable throughout the cold storage (days 9–12), particularly in the no dressing and garlic dressing treatments, ranging from 15.65 to 22.47 mg/L. These results suggest that there was a diffusion of glucosinolate from the broccoli stalk to the liquid, due to the increment of vegetable cell wall permeability in the fermentation process, and subsequent glucosinolates stabilisation under cold storage. On the other hand, in the mustard dressing treatments, the covering liquid showed lower glucosinolate content throughout the experiment because of the presence of myrosinase activity released from the mustard seeds during the milling process. It is important to highlight the glucoerucin and 4-methoxiglucobrassicin content in the covering liquid in all treatments, except in the mustard dressing which had the lowest glucoerucin content. Ciska et al. (2021) reported a similar diffusion in a fermented cabbage juice, finding a degradation as they obtained a juice by the maceration of shredded cabbage leaves and the release of endogenous myrosinase that degraded the glucosinolates in isothiocyanates and glucobrassicin breakdown such as ascorbinogen and indole-3-carbinol, with reported positive health effects.

3.6. Sensory evaluation

Fermented broccoli stalk slices improved the global acceptability in all treatments ($p < 0.001$) (Table S3). In general, the panellists reported better acceptability in the treatments with no dressing or garlic dressing, except at the end of the cold storge when the mustard dressing acceptability increased significantly to 7.33 ± 0.33, and differences among type of dressing were not statistically significant. The increase in LAB, accompanied by the TA and the drop in pH correlated with an increase in "pickled" aroma and taste, and a balanced saltiness and sourness (data not shown) which improved the global acceptability. Similar results were reported with high scores in flavour, texture, aroma, and acceptability in fermented brassica derivatives such as sauerkrauts and mustard greens (Satora, Skotniczny, Strnad, & Piechowicz, 2021). The initial broccoli stalk texture was 8.75, with no significant change at day 12. The fermentation process modified the typical raw broccoli aroma and taste, which had practically disappeared by day 3 in all treatments (data not shown); this was well received by the panellists. As the main result, the fermentation of broccoli stalk slices with or without dressing achieved a good global sensory quality.

4. Conclusions

This study presents a pickled food using vegetable by-products, in this case, broccoli stalks were naturally fermented in brine, obtaining a high variety and content in lactic acid bacteria, and bioactive compounds such as glucosinolates and phenolic compounds, which were identified and quantified through LC-ESI-MS/MS for monitoring the bioactive changes during food processing. On day 3, the fermented stalks showed a high content in bioactive compounds such as glucoerucin and indolic glucosinolates as glucobrassicin, 4-methoxy-glucobrassicin and 4-hidroxy-glucobrassicin, and phenolic acids and flavonoids such as sinapic acid, 4-O-feruloyl quinic acid and quercetin-3-O-diglucoside. The mustard dressing provided the highest phenolic content, principally in sinapic acid, increasing the functional potential of this product. On day 6, the broccoli stalks reached the peak level of health lactic acid bacteria and this status remained stable until day 12 at 4 °C, with *Latilactobacillus sakei* being the most predominant lactic acid bacteria, followed by *Lactobacillus curvatus*, *Leuconostoc kimchi* and *Leuconostoc carnosum*, providing immunomodulatory and metabolic health benefits.

This is an example of the revaluing of by-products that promote a circular economy and improve efficiency and profitability in the food sector through the design of a functional food and novel vegan product that contributes to diversity in the marketplace and provides a new taste and health benefits. Industrial scaling should be studied in future experiments.

CRediT authorship contribution statement

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

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.

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.lwt.2022.113915.

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