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Characterising the functional potential of fermented blueberry juice and its impact on the gut microbiota in an *ex vivo* colon model

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ABSTRACT

Blueberries, widely recognized as a "superfood," are rich in bioactive compounds whose functionality may be further enhanced through fermentation. This study investigated the nutritional and functional potential of blueberry juice fermented with *Leuconostoc mesenteroides* VUCC R-037 (or LM2), a native isolate, and *Lactiplantibacillus plantarum* DSM 25710 (or LP09), an industrial probiotic strain. Fermentation kinetics, microbial viability, and metabolic activity were monitored by microcalorimetry and selective plating, while compositional and functional changes in the fermented blueberry juice (FBJ) (such as total phenolics, antioxidant capability, putative cytotoxicity) were assessed through HPLC, chemical assays (FRAP), and *in vitro* cellular models (MTT and cytochrome *c* assay). Strain-specific contributions shaped the final product with LM2 promoting stronger acidification and acetic acid production, while LP09, favoured lactic acid accumulation and increased ferric reducing antioxidant capacity. Additionally, LM2 enhanced radical scavenging activity with co-cultures revealing complementary effects. FBJ supported probiotic viability, showed no cytotoxicity in CaCo-2 intestinal epithelial cells, preserved epithelial barrier integrity, and mitigated oxidative stress. Assays for impact of the FBJ on the gut microbiota was carried out in the micro-Matrix™ *ex vivo* distal colon model, where it selectively reduced *Clostridium perfringens* and *Escherichia* spp., suggesting a role in beneficial gut microbiota modulation. Overall, fermentation with native and probiotic lactic acid bacteria improved the biochemical, functional, and cytoprotective profile of blueberry juice, highlighting its promise as a novel functional beverage. While further clinical validation is forthcoming, these results strengthen the case for fruit-based fermented products as innovative vehicles to deliver both health-promoting microbes and bioactive compounds to consumers.

1. Introduction

Over millennia, fermented foods have become global staples such as bread, dairy products (e.g., yogurt, cheese, kefir), fermented meats (e.g., sausages), and beverages like coffee and cocoa, due to their cost-effectiveness, economic potential and sustainable attributes. (Gänzle, 2022).

Recently, fermented fruit products have gained popularity in the

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food industry due to their enhanced flavour and potential to impart health benefits (Gangakhedkar et al., 2025). Among fruits, blueberries have attracted particular attention because of their high levels of micronutrients and health-promoting phytochemicals, especially polyphenols. Often labelled as "superfoods," blueberries are rich in essential minerals, vitamins, fatty acids, and dietary fibre (Miller et al., 2019). Additionally, they contain a wide array of bioactive compounds, particularly phenolic compounds and organic acids (Li et al., 2021) that have been associated with anti-inflammatory properties (Yang et al., 2021), improved cognitive function (Philip et al., 2019), and potential protective effects against cardiovascular disease and certain cancers (Yang et al., 2019). However, fresh blueberries are highly perishable, with their use limited by seasonality, rapid spoilage, and a short shelf life (Zhang et al., 2021). Fermentation has thus emerged as a valuable strategy to transform blueberries into functional foods. The process, often involving Lactic Acid Bacteria (LAB), not only extends their shelf life but also increases the bioavailability of polyphenols and antioxidants, thereby enhancing antioxidant activity and sensory properties (Sivapragasam et al., 2023). Several recent studies have also demonstrated the health-promoting potential of fermented blueberries. For instance, rats fed with fermented blueberry products exhibited improved liver protection and reduced oxidative stress, with these effects attributed to phenolic compounds released during fermentation (Xu et al., 2013). Furthermore, blueberries fermented with tannase-producing LAB showed greater antiproliferative activity against human cervical carcinoma cells than raw blueberries, with phenolic acids, such as protocatechuic acid and catechol, playing key roles (Mallet et al., 2023; Ryu et al., 2019).

Fermentation with probiotic microorganisms, defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014), can enhance the antibacterial, antioxidant, and antidiabetic properties of blueberry products. For example, Zhong et al. (Zhong et al., 2021) showed that blueberry juice fermented with *Lactiplantibacillus plantarum*, *Saccharomyces cerevisiae*, and *Hendrickxia coagulans* strains had lower sugar content, higher organic acid levels, and exhibited antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*. Similarly, Wu et al. (Wu et al., 2021) reported increased radical scavenging activity in blueberry juice fermented with *Lpb. plantarum*, *Streptococcus thermophilus*, and *Bifidobacterium bifidum* strains (Wu et al., 2021).

In the present study we have built on existing research on fermented blueberry juice by developing a novel fermented blueberry juice (FBJ) beverage with improved nutritional qualities by combining, for the first time, the potential of a native strain, *Leuconostoc mesenteroides* VUCC R-037 (hereafter referred to simply as LM2), isolated from spontaneously fermented blueberry and dominant through fermentation, and an industrial strain, *Lactiplantibacillus plantarum* LP09 (DSM 25710), already marketed as a probiotic strain (Probiotical S.p.A). The chemical composition of the FBJ was monitored throughout the fermentation process, with a focus on antioxidant activity and the evolution of phenolic compounds; further FBJ was also investigated as a mode to deliver other probiotic strains (i.e. *Bifidobacterium animalis* supsp. *lactis*). Finally, the health-promoting potential of the FBJ was evaluated through determination of its impact on the gut microbiota in an *ex vivo* human distal colon model as well as through in vitro cytotoxicity and antioxidant assays.

2. Methods

2.1. Single and mixed strain blueberry juice fermentation

The commercial blueberry juice (BJ, 500 mL, 100% blueberry juice obtained from ca. 1800 blueberries) was purchased at a local retailer (Esselunga S.p.A., Milan, Italy). The juice was heated until boiling and then immediately cooled in ice to remove the potential presence of indigenous microorganisms. Before the addition of strains used for fermentation, the juice was diluted with sterile water (50% *v*/v) and the pH adjusted to ~6 with 4 M NaOH solution to mimic the industrial setting, where food-grade NaOH is commonly employed to control industrial fermentation. *Leuconostoc mesenteroides* strain VUCC R-037 (deposited in the Verona University Culture Collection) (hereafter referred to simply as LM2), previously isolated from blueberries, and *Lactiplantibacillus plantarum* LP09 (DSM 25710), (hereafter referred to as simply LP09), a commercial probiotic strain provided by Probiotical Spa (Italy), were used for single and co-culture fermentations. The microbial inoculum was added after washing the cells twice with physiological saline solution (0.9% NaCl). In co-culture (or mixed) fermentations, the two strains were added in two inoculum ratios, 1:1 and 10:1 (LM2 and LP09, respectively), at an initial microbial load of 6 Log colony forming units (CFU)/mL. The 10:1 ratio was chosen to promote LM2 growth while minimizing the overgrowth of LP09. Fermentation trials were conducted for 48 h at 37 °C and pH was monitored throughout the fermentation.

2.2. Isothermal microcalorimetric analysis

Microcalorimetric systems provide real-time, non-destructive monitoring of fermentation processes by measuring heat flow generated by microbial metabolic activity (Cuenca et al., 2017). According to a basic model, the amount of heat generated is theoretically proportional to the consumption of substrates, the production of biomass, and the release of byproducts (Braissant et al., 2013; Braissant et al., 2015). After a 24-h overnight inoculation of *Lpb. plantarum* LP09 and *L. mesenteroides* LM2 in appropriate media, a 10-fold dilution of the bacterial suspensions was prepared in peptone water. More specifically, single fermentations were conducted with 6 Log CFU for each inoculum (LP09 or LM2), while mixed fermentations were conducted with two different inoculations (6 Log CFU and 7 Log CFU) maintaining the ratio 10:1 (LM2: LP09) between the two inoculations. The isothermal microcalorimeter plate was prepared by adding 400 μL of unfermented BJ containing the inoculum for both single and mixed fermentations. The vials were then placed into a calScreener™ isothermal microcalorimeter (Symcel AB, Sweden) following the manufacturer's guidelines. Kinetic heat flow was monitored over a 48-h period using calView 2.0 software (Symcel AB, 2023), which generated heat flow curves (Morazzoni et al., 2024). To enable quantitative comparison of microbial growth kinetics, several parameters such as total accumulated heat, the metabolic rate (indicated by the total heat released), the maximum metabolic rate (the peak heat flow value detected), the time to activity (i.e., the time when the first metabolic signal is detected) and time to peak (i.e., the duration required to reach the maximum metabolic rate) were extracted using the online analysis tool calData (Morazzoni et al., 2024). To assess bacterial growth under standardized conditions, de Man Rogosa Sharpe (MRS, Oxoid Ltd., Thermofisher Scientific Waltham, MA, USA) broth was used as a control medium and unfermented BJ was used as negative control.

2.3. Strain survival

After 48 h of fermentation, the FBJ was stored at 4 °C for 28 days. Strain survival was monitored every three days through selective counts on Homofermentative-Heterofermentative Differential (HHD) medium (McDonald et al., 1987) where *L. mesenteroides* LM2 showed white colonies (heterofermentative metabolism) while *Lpb. plantarum* LP09 showed green colonies (homofermentative metabolism). *Bifidobacterium animalis* BA, a commercial probiotic strain (Probiotical Spa), was also added to check if FBJ could be also a delivery vehicle for probiotics. To selectively count *B. animalis*, MRS medium (Thermofisher Scientific Waltham, MA, USA) supplemented with 32 mg/L gentamycin was used, in accordance with EFSA guidelines (European Food Safety Authority (EFSA), 2018).

2.4. Chemical analysis of the fermented blueberry juice

Lactic, acetic, and citric acid content and sugar concentration of FBJ were evaluated by HPLC analysis; BJ was employed as a control. Samples were separated and quantified for glucose and fructose using an Aminex HPX 87C fixed ion resin column. The column was set at 60 °C and the eluent 4.5 mM H_2SO_4 was run at 0.5 mL/min, and the sugars were detected using a refractive index detector with a sensitivity set at 512, an internal temperature set at 35 °C and a positive polarity. Citric, lactic, and acetic acids were quantified using a Rezex ROA-Organic Acid H^+ (8%) ion exclusion column. The column was set to 55 °C and the eluent 2.5 mM H_2SO_4 was run at 50 μL/ min. Before analysis samples were centrifuged at 10,000 rpm for 5 min, and the supernatant was diluted 100-fold to be in the range of standards concentrations.

2.5. Determination of antioxidant capabilities

To assess FBJ antioxidant capacities, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was carried out according to Brand-Williams et al. (1995) with some modifications. Briefly, 1 mL of FBJ was centrifuged at 10,000 rpm for 5 min, and a 10 μL aliquot of the supernatant was placed in a 96-well microplate, containing 190 μL of DPPH solution in methanol (initial absorbance at 515 nm c.a. 1.00). The decrease in absorbance at 515 nm was determined by monitoring the absorbance changes in cycles of 1 min during 10 min using a microplate reader (Biotek Synergy HT, Com3, Sunnyvale, California, USA). Ascorbic acid solution was used to make the calibration curve calculating Antioxidant Radical Absorbance percentage (ARA%).

The ARA% was calculated according to the following equation (expressed as vitamin C equivalent antioxidant capacity-VCEAC in mg of vitamin C/g)

$$ARA\% = 100 \times \left(1 - \frac{A_{ss}}{A_0}\right)$$

where A_{SS} is the absorbance of the solution in a steady state, and A_0 is the absorbance of the DPPH solution before adding the antioxidant.

The Ferric Reducing Antioxidant Capacity (FRAP) assay was performed to evaluate the reducing power of FBJ according to the modified version of Tao et al. (2016). Briefly, the FRAP reagent was freshly prepared by mixing 10 mmol/L 2,4,6-Tripyridyl-s-Triazine solution in 40 mmol/L HCl, 300 mmol/L acetate buffer (pH 3.6) and 20 mmol/L $FeCl_3{\cdot}6H_2O$ in a ratio of 1:10:1. For each assay, 1 mL of FBJ was centrifuged at 10,000 rpm for 5 min, and a 10 μL aliquot of the supernatant was placed in a 96-well microplate, containing 190 μl of FRAP reagent. The mixture was incubated at 37 °C in the dark for 8 min. The absorbance was measured at 593 nm. $FeSO_4$ solution was used to make the calibration curve. The results were expressed as mmol of $FeSO_4$ equivalents per litre of FBJ.

2.6. Total phenolic content analysis

The Folin-Ciocalteu assay quantified the total phenols in FBJ according to the modified version of Singleton and Rossi (1965). Briefly, FBJ was centrifuged at 10,000 rpm for 5 min, then 200 μL of the supernatant was mixed with 650 μL of 1 N Folin-Ciocalteu reagent. 3.25 mL of Na_2CO_3 (0.2 g/ mL) solution was added after 5 min of incubation at room temperature. The volume was adjusted to 5 mL with water, allowed to cool down in ice for 1 min, and the absorbance was measured at 765 nm. Gallic acid was used to make the standard curve to express results as gallic acid equivalents (μg/mL).

2.7. Cytotoxicity evaluation on CaCo-2 cells

CaCo-2 cells were obtained from ATCC and subcultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin, and maintained at 37 °C in a humidified atmosphere with 5% CO_2. All reagents were purchased from Euroclone (Milan, Italy).

The cytotoxicity capacity was evaluated in both BJ and FBJ obtained with LM2 and LP09 strains at a 10:1 ratio. Cytotoxicity was assessed using the MTT assay (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), as described by Ma et al. (2013), on CaCo-2 cell line following treatment with FBJ. Undifferentiated CaCo-2 cells were cultured in a 24-well plate in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, USA). Prior to treatment, culture medium was replaced by fresh DMEM. After removing the supernatant, fresh medium containing either FBJ or BJ at concentrations ranging from 0.1% to 1% (*v*/v) was added to each well to a final volume of 1 mL. This range of FBJ/BJ concentrations was selected to avoid the technical interferences of pigmented and polyphenol-rich matrices (such as blueberry juice) with colorimetric viability assays. Higher concentrations cause significant optical and chemical interference, making the assay unreliable.

The plate was incubated at 37 °C for 24 h. After the incubation, the supernatant was removed, and the cell layer was washed with Dulbecco's Phosphate Buffered Saline (DPBS). The MTT solution (5 mg/mL in DPBS) was then added at a 10% of the well volume, and the plate was incubated for 1.5 h at 37 °C. After incubation, the supernatant was discarded, the cells were washed with DPBS, and 100 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm.

2.8. Barrier protection assay on CaCo-2 cells

For spontaneous differentiation, CaCo-2 cells were seeded onto polycarbonate filters (12 mm diameter, 0.4 μm pore size, Millipore) at a density of 3×10^5 cells/cm^2 in complete medium added to both apical and basolateral compartments. Cells were allowed to differentiate for 21 days, with medium changes twice a week. Following differentiation, cells were exposed in dynamic culture conditions using the LiveBox1 system (LB1, IVTech), with BJ and FBJ (LM2 10: LP 1) in the presence of 1 μg/mL lipopolysaccharide (LPS). Treatment with LPS alone served as a positive control. The perfusion flow rate was maintained at 150 μL/min (Colombo et al., 2023). Transepithelial electrical resistance (TEER) was measured prior to stimulation and after 5 h of treatment, following the protocol described by Wallace et al. (2014) in order to assess changes in cellular permeability. The final values are expressed as Ω/cm2 on the basis of the equation: TEER = (R-R_B) x A, where R is the resistance of filter insert with cells, R_B is the resistance of the filter alone and A is the growth area of the filter.

2.9. Cytochrome C assay

The cytochrome *c* assay was performed to assess the oxidative damage of CaCo-2 cell lines treated with FBJ (10,1 LM2, LP09) (Catino and Miceli, 1988). Undifferentiated CaCo-2 cells were cultured in a 24-well plate, and the culture medium (DMEM supplemented with 10% FBS) (Thermo Fisher Scientific, Waltham, MA, USA) was replaced with fresh medium. After removing the supernatant, a fresh medium containing either FBJ blueberry juice (BJ) or pH-corrected BJ (pH 6) was added at concentrations of 10% to achieve a final volume of 1 mL per well. A single pH-adjusted BJ (pH 6) was used as a control to account for general acidity effects without extensively altering the juice matrix, which could introduce confounding changes in phenolic composition and redox properties. The plate was then incubated at 37 °C for 24 h. Following incubation, the supernatant was centrifuged and supplemented with 10% cytochrome *c* solution (10 mg/mL) for a 40-min incubation at 37 °C. Absorbance was subsequently measured at 550 nm.

2.10. Ex vivo distal colon model assays

The micro-Matrix™ bioreactor platform (Applikon) was used in this

study and served as an *ex vivo* human distal colon model. The mini bioreactor system consists of a 24-well cassette hermetically sealed to avoid contamination and oxygen access, placed in a fermentation system with monitored pH, dissolved oxygen (DO%), and temperature. The associated micro-Matrix HMI software automatically modifies all analysed parameters to maintain the desired conditions, such as pH 6.8, DO at 0%, and 37 °C, with agitation of the cassette at 275 rpm to avoid pellet formation. The colon model experiments were conducted for 24 h in this study, taking samples at 0 h (T0: start of experiment) and 24 h (T24: end of experiment); these samples were used for downstream DNA sequencing and for quantifying the total number of bacterial cells in each of the samples. In order to prepare the distal colon model experiments, a batch of frozen standardized inoculum (FSI) faecal slurry was prepared as described previously (Mathur et al., 2023).

The FSI was derived from pooled faecal samples from seven volunteers and was approved by the Clinical Research Ethics committee (CREC) in Cork, Ireland as part of study APC179. Participants aged between 18 and 65 yrs. were recruited. Volunteers who had taken antibiotics, and/or any other medication deemed to have an impact on the composition of the gut microbiota in the three months before sampling formed part of the exclusion criteria.

To understand and evaluate the effects of FBJ on the gut microbiome, Fooks & Gibson Faecal Fermentation Media (FFM) was used as a basal medium for the distal colon model experiments as described previously (Mathur et al., 2023). The FFM:FSI mix was added to a few wells in a micro-Matrix plate and a 16-h adaptation period was introduced by conducting a blank micro-Matrix experiment, simulating the environmental conditions in the distal colon with a pH of 6.8, temperature of 37 °C and dissolved oxygen concentration of 0 units. After this 16-h adaptation period, 0.8 ml of the FFM:FSI mix was taken and added to each of the wells of the micro-Matrix cassette. This was topped up with 2.7 mL of fresh double-strength FFM. Finally, 3.5 mL of the FBJ was added to the wells, thereby rendering the final concentration of the FFM to a 1× concentration. *Bif. animalis* (BA) was added to the BJ to determine the effect of the presence of the cells, and BA was added to FBJ fermented with LM2 + LP09 (10:1). The latter sample was heat-treated to determine if the results were influenced by live or dead cells. The micro-Matrix experiment was conducted for 24 h. All samples were tested in triplicate.

2.11. 16S rRNA amplicon sequencing

DNA was extracted from micro-Matrix samples using the QiaAmp PowerFecal Pro kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The concentrations of the DNA samples were then quantified using the Qubit 1× dsDNA High-Sensitivity DNA kit (Thermo Fisher, MA, United States) and a Qubit 4.0 fluorometer (Thermo Fisher, MA, USA) and subsequently normalised to 5 ng/μL as a starting template concentration. 16S rRNA amplicon sequencing libraries were subsequently prepared as described previously (Koc et al., 2024). Concentrations of the libraries were determined as mentioned above and all libraries were pooled to a final concentration of 4 nM. Following this, sequencing was performed using a P1 600-cycle (2 × 300 bp) reagent kit (Illumina, California, USA) on the Illumina NextSeq 2000 sequencer according to manufacturer's guidelines at the Teagasc Next Generation DNA Sequencing Facility.

2.12. Flow cytometry

To understand the gut microbiota composition in the micro-Matrix samples in terms of absolute abundance, the total number of cells in each sample was quantified using flow cytometry (BD Accuri™ C6). Flow cytometry was conducted as described previously (Mathur et al., 2023). Briefly, 300 μL aliquots of relevant samples from the micro-Matrix experiments and time points were autoclaved at 121 °C degrees for 15 mins to heat-kill all bacterial cells. These heat-killed cells were stained with Syto 9 and Propidium Iodide (BacLight Bacterial viability kit) as previously described (Mathur et al., 2018; Mathur et al., 2016). A quadrant style gating strategy was used to categorise the bacterial cell population into live, stressed/injured, dead and fragmented cells. The total cell population with cells residing in the 'Dead' and 'Fragmented' quadrants were used to determine the total bacterial cell numbers in terms of Active Fluorescent Units (AFU). These total bacterial cell numbers were combined with the relative abundance data to calculate absolute abundance values as previously described (Mathur et al., 2023).

2.13. Bioinformatic analysis

16S rRNA amplicon sequencing data for all samples were checked for quality using FastQC and MultiQC (Andrews, 2010; Ewels et al., 2016). 16S rRNA sequences were then processed using Qiime2 (Bolyen et al., 2019). Briefly, demultiplexed 16S rRNA sequences were denoised using the Qiime2 implementation of DADA2 (Callahan et al., 2016), with truncation and trimming of the sequences carried out using the following arguments (−-p-trim-left-f 10 –p-trim-left-r 10 –p-trunc-len-f 290 –p-trunc-len-r 220). Amplicon sequencing variants (ASVs) thereby identified using DADA2 were then taxonomically classified using the SILVA SSU r138.2 release (Pruesse et al., 2007) in QIIME2 and annotations for chloroplasts and mitochondria removed. Compositional taxonomic data for samples were then extracted for each taxonomic level and graphed as relative abundances with ggplot2 in R (Wickham, 2016). Taxonomic clades not present at >1% relative abundance in at least one sample were not shown separately during graphing. Absolute abundance plots merging relative abundance composition and absolute cell counts were also prepared in ggplot2 in R (v.4.3.1).

2.14. Statistical analysis

All statistical analyses were performed using R. Data normality was assessed using Shapiro-Wilk test. For normally distributed data, one-way ANOVA followed by Tukey's HSD post-hoc test was used. Data are presented as mean ± SD of three replicates. The sample size consisted of three biological samples and one technical replicate for each analysis, except for organic acids analysis, where three technical replicates of the same biological sample were considered.

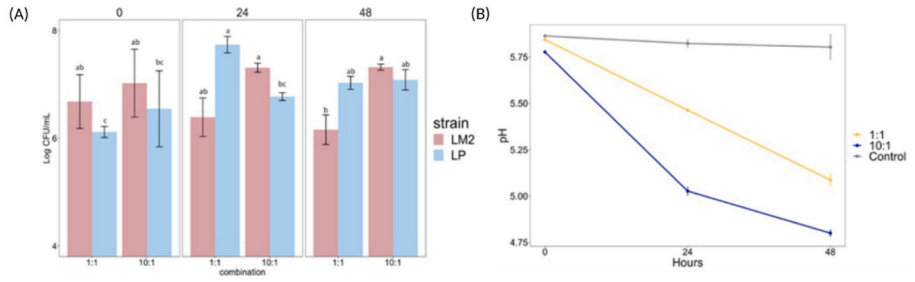
2.15. Data deposition

Raw 16S rRNA amplicon sequencing data generated in this study was submitted to the European Nucleotide Archive under BioProject PRJEB96788 (secondary accession: ERP179402).

3. Results

3.1. Production of probiotic fermented blueberry juice

Blueberry juice (BJ) fermentation was conducted with *Leuconostoc mesenteroides* LM2 and *Lactiplantibacillus plantarum* LP09 either as single inoculum or at 1:1 and 10:1 ratios, respectively. The presence of the two strains was monitored over a 48-h fermentation period in BJ through plating in HHD medium at beginning (0 h), middle (24 h) and end of fermentation (48 h) (Fig. 1). In single fermentation trials, LM2 strain reached 7.39 ± 0.11 Log CFU/mL, and a greater efficacy in reducing pH achieving a final pH of 4.86 ± 0.01, while LP09 reached 7.97 ± 0.10 Log CFU/mL after 48 h with a final pH of 5.33 ± 0.02 (Supplementary Fig. S1). In mixed fermentation trials, the initial inoculum concentration ranged between 6 and 7 Log CFU/mL, with a slightly higher count for LM2 in the 10:1 inoculum ratio. In the 1:1 ratio, LP09 outgrew LM2, diminishing the pH value down to 5.09 ± 0.03 and increasing the viable cell counts to 7.04 ± 0.12 Log CFU/mL. At the end of the fermentation (48 h), both strains showed the same microbial count (approximately 7



a) b)

Fig. 1. Mixed fermentation in BJ with an initial microbial ratio of 1:1 and 10:1 for LM2 (VUCC R-037) and LP (LP09) strains respectively: a) strains' microbial kinetic assessed on MRS medium; b) pH trend during fermentation. The sample size consisted of three biological samples and one technical replicate.

Log CFU/mL, *p*-value = 0.13). The contribution of LM2 in the 10:1 fermentation ratio was evident from the significantly lower pH value (4.8 ± 0.01) observed after 48 h, which could be attributable to the heterofermentative metabolism of *L. mesenteroides* and its capacity to produce acetic acid.

In terms of viability, strains remained viable for 28 days at 4 °C with a stable cell count of approximately 7 Log CFU/mL for the LM2 and LP09 strains. *Bifidobacterium animalis* BA also exhibited a good survival rate, though with a slight decrease of approximately 1 Log CFU. A pH decrease of approximately 0.4 units was observed in both FBJ samples, indicating residual microbial activity and metabolism (Supplementary Fig. S2).

The investigation of metabolic activity showed that both strains contribute to blueberry juice fermentation. The microcalorimetric analysis demonstrated distinct patterns based on the initial inoculum and strain. Specifically, LP09 displayed a higher metabolic activity, reaching a maximum heat peak of 18.99 μW after 7.43 h of incubation. In contrast, LM2 exhibited a slower metabolism, with a detectable peak of 11.80 μW achieved after 14.73 h (Fig. 2A). The trends observed in single-strain analyses were corroborated by co-inoculation trials (Fig. 2B). Notably, in the co-culture with a total inoculum of 7 Log CFU, LP09 exhibited a more pronounced contribution, with a peak of 14.66 μW, effectively overshadowing the calorimetric signal of LM2. At a concentration of 6 Log CFU, contributions from both strains were evident, with LP09 predominating for the initial 8 h, followed by a notable increase in the contribution from LM2, reaching a peak at 15.76 μW. Conversely, for single strain inocula, the total heat, as indicated by the area under the calorimetric curve, was comparable for both LM2 and

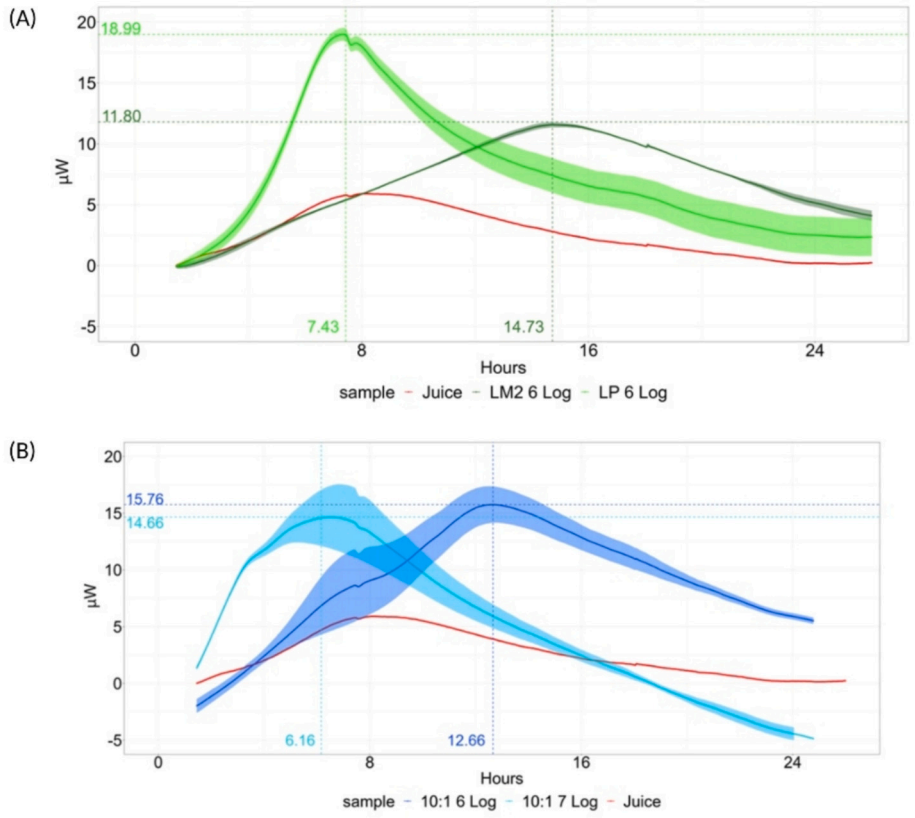


Fig. 2. Microcalorimetry heat flow profiles for (a) single-strain fermentation in unfermented BJ, and (b) mixed-strain fermentations with a 10:1 ratio of LM2 (VUCC R-037) to LP (LP09) strains. Microbial loads are expressed as colony-forming units (CFU) per well. The time to peak (indicated near the y-axis) and the maximum heat flow (indicated near the x-axis) achieved under each condition are reported in the respective plots. The sample size consisted of three biological samples and one technical replicate.

LP09. However, in co-inoculation scenarios, there was a significant difference in the total microbial load, with the 6 Log CFU co-inoculation generating a higher total heat production (Supplementary Fig. S3).

Overall, the calorimetric analysis indicated a slower metabolic rate for the LM2 strain in comparison to LP. This observation is further corroborated by the fermentation results of the individual strains.

3.2. Chemical analysis of the fermented blueberry juice

Organic acid and sugar content in FBJ samples were quantified by HPLC (Supplementary Table S1). In BJ, the total sugar concentration (glucose and fructose) was around 35 g/L. Glucose was consumed by consortia, with no difference between 1:1 and 10:1 ratio. On the other hand, fructose was only significantly consumed in the 10:1 fermentation (Fig. 3). This is consistent with the fructophilic behaviour of LM2, which was isolated from blueberries.

The organic acid quantifications were consistent with the pH evaluation during the fermentation. Citric acid was identified as the predominant organic acid in BJ, with its concentration remaining stable throughout the fermentation (between 2.18 and 2.68 g/L) (Supplementary Table S1). Acetic acid was produced exclusively by *L. mesenteroides* LM2 due to its heterofermentative metabolism, with concentrations ranging from 0.57 ± 0.05 g/L in the LM2 single-strain fermentation to 0.68 ± 0.03 g/L in the 10:1 ratio co-inoculum. This suggests a synergistic effect between the LM2 and LP09 strains in enhancing acetic acid production. Consequently, the 10:1 ratio fermentation achieved the lowest pH (4.81 ± 0.02), attributed to the significant concentrations of both lactic and acetic acid. Lactic acid production was primarily attributed to *Lb. plantarum* LP09 (2.19 ± 0.11 g/L), which produced 0.45 g/L more than the LM2 strain (1.74 ± 0.14 g/L), significantly increasing its concentration during mixed-strain fermentation at a 1:1 ratio (2.51 ± 0.19 g/L). This behaviour is likely due to the faster growth rate of the *Lb. plantarum* strain, resulting in its higher metabolic activity.

3.3. Antioxidant properties and phenolic composition of the FBJ

According to the DPPH assay (Table 1), LM2 fermentation slightly increased the antioxidant capacity of FBJ (10.83 ± 0.34 μg/mL) compared to BJ (10.14 ± 0.21 μg/mL), whereas LP09 had a negative effect, reducing the antioxidant capacity of the juice (7.43 ± 0.86 μg/mL). This result was confirmed in mixed fermentations, where the fermentation in a 1:1 ratio resulted in a decrease of the antioxidant capacity (7.68 ± 0.19 μg/mL). In the fermentations with a 10:1 ratio, it remained almost comparable to the unfermented blueberry juice (9.08 ± 0.35 μg/mL). The antioxidant capacity, as assessed by the FRAP assay (Table 1), was slightly higher in the LP09-fermented juice (23.76 ± 1.9 μg/mL) compared to the BJ (21.44 ± 0.49 μg/mL), and this trend was also observed in the 1:1 mixed fermentation (22.4 ± 0.75 μg/mL). However, LM2 fermentation did not significantly decrease the ferric reduction capacity (21.85 ± 0.54 μg/mL).

Table 1
Antioxidant activity (DPPH and FRAP) and total phenolic content (Folin-Ciocalteau) of FBJ in single and mixed strains fermentation.

Assay	Control	LP	LM2	1:1	10:1
DPPH[1]	10.14 ± 0.21^{ab}	7.43 ± 0.86^{c}	10.83 ± 0.34^{a}	7.68 ± 0.19^{c}	9.08 ± 0.35^{b}
FRAP[2]	21.44 ± 0.49^{ab}	23.76 ± 1.9^{a}	21.85 ± 0.54^{ab}	22.4 ± 0.75^{ab}	19.91 ± 1.01^{b}
Folin-Ciocalteu[3]	58.83 ± 0.69^{b}	58.95 ± 0.77^{b}	55.95 ± 1.04^{a}	56.86 ± 0.22^{a}	53.04 ± 0.38^{c}

[1] Vitamin C equivalent antioxidant capacity (VCEAC) (μg/mL).
[2] mmol $FeSO_4$ g/L;
[3] gallic acid equivalents (μg/mL). a,b,c: indicates statistical significance.

Finally, the Folin-Ciocalteu assay showed that the phenolic content marginally decreased across all strains and ratios (Table 1), except for LP09, which maintained the same phenolic content of the unfermented juice (58.95 ± 0.77 μg/mL and 58.83 ± 0.69 μg/mL, respectively).

3.4. Cytotoxicity evaluation and in vitro oxidative damage on CaCo-2 cells

Cytotoxicity assays conducted by following the tetrazolium salt method using the MTT technique showed that both BJ and FBJ (LM2: LP09; 10:1) did not affect the viability of undifferentiated CaCo-2 cells after 24 h incubation at 37 °C (Supplementary Table S2). To assess the protective effect on cell barriers, TEER assays was performed, which quantified the electrical resistance across a CaCo-2 cell monolayer after induced damage by treatment with lipopolysaccharide (LPS) at 1 μg/mL. Cell treated with LPS resulted in a reduction in the TEER values, indicating a disruption of the cell layer. The addition of both BJ and FBJ (LM2: LP09; 10:1) to LPS-treated cells increased the TEER values, indicating an improved integrity of the tight junctions and thus a protective effect on cell lines (Supplementary Fig. S4).

The oxidative damage of CaCo-2 cells treated with FBJ (LM2: LP09; 10:1) and BJ was then evaluated using the cytochrome *c* assay, both with and without the addition of 400 μL H_2O_2 in 1 mL final volume. To mitigate potential bias from pH variation, a sample of pH-adjusted BJ

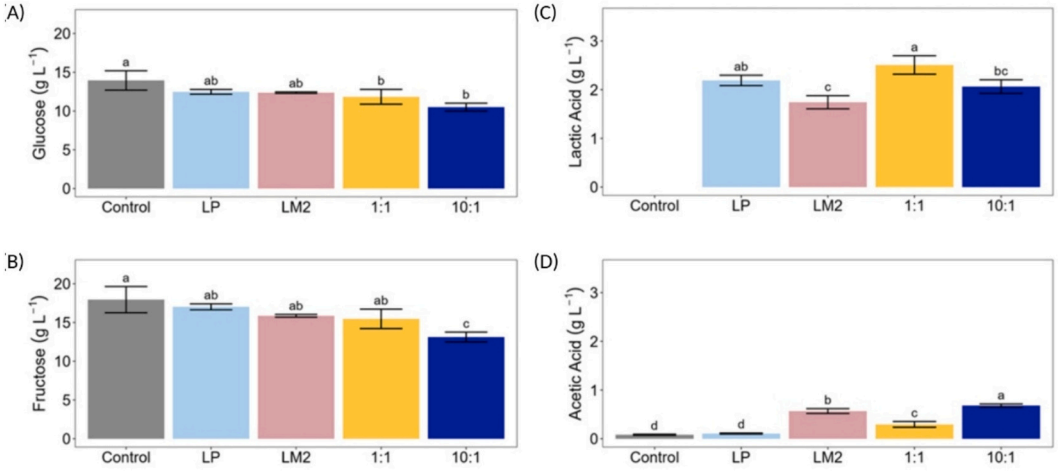


Fig. 3. Sugars and organic acid content of FBJ in single and mixed strains fermentations. A: glucose (g/L), B: fructose (g/L), C: lactic acid (g/L), D: acetic acid (g/L). LM2: VUCC-037, LP: LP09. Three technical replicates of the same biological sample were considered.

(pH 6) was included in the experimental conditions. The inclusion of a pH-adjusted sample addressed the potential confounding effect of pH variation on the results, ensuring that the antioxidant effects observed were not simply due to pH differences between samples. Under basal conditions (Fig. 4A), FBJ demonstrated notable antioxidant capacity compared to the negative control, effectively limiting the presence of reactive oxygen species. However, its antioxidant activity was slightly lower than that of the commercial juice and the pH-adjusted juice, with no significant difference observed between the latter two. These findings were corroborated in the presence of 400 μL H_2O_2 (Fig. 4B), where cytochrome *c* in the FBJ-treated sample showed significantly less oxidation than in the positive control. Nonetheless, as in the basal conditions, the antioxidant capacity of FBJ was marginally lower than that of the commercial and pH-adjusted juices.

3.5. Ex vivo distal colon model assays

The micro-Matrix™ bioreactor platform as an *ex vivo* model of the human distal colon was used to assess how FBJ (LM2:LP09; 10:1) impacts the composition of the gut microbiome. The 16S rRNA amplicon-based sequencing approach revealed that every substrate elicited the growth of *Klebsiella* spp. as indicated by the high relative abundance (approx. 50%) in every sample, including the positive control and negative control (Fig. 5A). This phenomenon of coliform blooms has been reported in recent studies (Mathur et al., 2023; Mukherjee et al., 2025) and can render results from the micro-Matrix challenging to be fully assessed and thus they should be interpreted with caution. Nonetheless, since all samples were afflicted with these *Klebsiella* blooms in these experiments, it was still possible to gain certain insights into the effects of FBJ relative to negative controls. It was noteworthy that both LM2 and LP09, employed for the fermentation of blueberry juice, as well as BA, inoculated post-fermentation, were consistently detected in the corresponding samples. A similar trend was observed in both viable and heat-treated fermented blueberry juice samples. This provided biological relevance to the relative abundance data despite the samples showing *Klebsiella* blooms.

While relative abundance is useful and provides significant insights, the metric does have some inherent limitations. Most notably, it provides insights into compositional analysis by indicating the relative percentages of each genus or species. However, it does not provide information about the exact numbers of these genera or species of interest (for, e.g., a certain species might have decreased somewhat in relative abundance over time but may have increased in total population numbers over the same period of time). Therefore, in order to gain more precise insights, a flow cytometry approach was adopted to quantify the precise numbers of bacterial cells which were present in each of the samples from the micro-Matrix experiments. Flow cytometry cell count numbers were then merged with relative abundance data to calculate absolute abundance of certain taxa and genera of interest. This analysis revealed precise differences in the compositional changes in the colonic microbiome which were induced by the fermented blueberry juice samples. After 48 h, only two *taxa* (*Clostridium perfringens* and *Escherichia* spp.) exhibited a significant change (Fig. 5B). *Clostridium perfringens* showed a decrease in FBJ treated with LM2 and LP09, both with and without the addition of BA. Similarly, *Escherichia* spp. decreased across all FBJ-treated samples, while no change was observed in the control, indicating a potential inhibitory effect of FBJ.

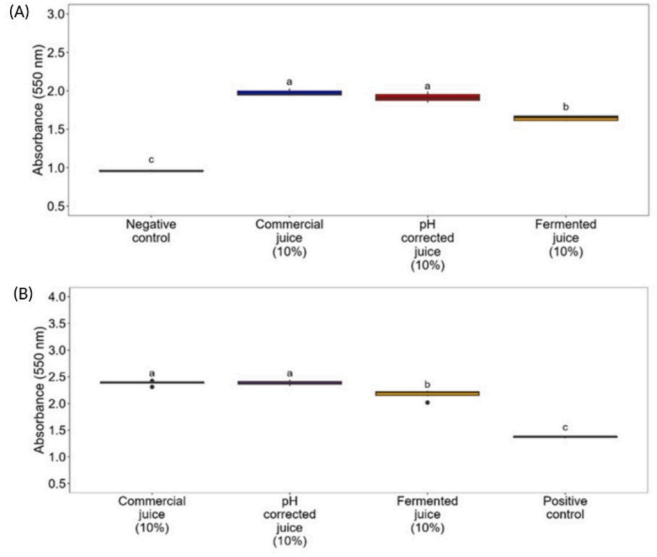


Fig. 4. Cytochrome assay on both commercial juice and fermented juice (LM2:LP; 10:1). a) negative control in DMEM medium and treatment conditions, b) all samples were treated with 400 μL H_2O_2 including positive control. LM2: VUCC R-037, LP: LP09. The sample size consisted of three biological samples and one technical replicate.

4. Discussion

Fermentation is an age-old technique that remains highly relevant for enhancing food preservation, nutrition, and functionality. Withing this context, Lactic Acid Bacteria (LAB) play a key role in improving both the sensory and health-promoting properties of fermented foods.

In this study, the strains *Leuconostoc mesenteroides* LM2, previously isolated from blueberries (data not shown) and *Lb. plantarum* LP09, a probiotic industrial strain (Probiotical Spa), were used as fermentative agents of the blueberry juice (BJ), aiming to optimize its nutritional and sensorial qualities as well as improving its health benefits. *Bifidobacterium animalis* BA, another probiotic industrial strain (Probiotical Spa), was added in the final product to evaluate the suitability of the fermented beverage to deliver live strains to the final consumer. Fermentation kinetics showed that the *L. mesenteroides* LM2 could represent a good starter culture for BJ fermentation as the fermented beverage reached the lowest pH (4.86 ± 0.01), likely due to its heterofermentative metabolism and acetic acid production, with a final cell count of 7.39 Log CFU/mL. This is the first study in which a native blueberry *L. mesenteroides* strain is used for BJ fermentation, building on previous findings showing the species potential in fermenting other fruit juices (Zheng et al., 2014; Fessard and Remize, 2017; Lee et al., 2016; Cassimiro et al., 2023). *Lactiplantibacillus plantarum* LP09 reached about 8 Log CFU/mL, though this was lower than levels reported in previous studies with other strains and with higher initial inocula (Li et al., 2021; Zhang et al., 2021). In co-culture fermentation (1:1 ratio), the total microbial load reached 7.04 ± 6.47 Log CFU/mL, meeting the recommended probiotic threshold for potential health benefits (Aragon-Alegro et al., 2007), though pH reduction was less marked compared to other studies using more acid-tolerant strains (Liao et al., 2023).

Fermentation dynamics and single strains contribution were monitored through isothermal microcalorimetry, which revealed notable differences in the metabolic activity of LP09 and LM2. LP09 showed faster and higher peak in heat production (15.76 μW after 7.43 h), indicating greater metabolic activity compared to LM2, which was further observed in co-culture fermentation at high inoculum levels (7 log CFU). However, in co-culture at lower inoculum levels (6 Log CFU), both strains contributed more evenly, with LM2 showing delayed but increased activity after LP09's initial peak. Despite differing kinetics, both strains produced similar total heat in single cultures, suggesting comparable overall metabolic output under optimal conditions. The enhanced total heat in co-inoculation at 6 Log CFU suggests potential synergistic interactions that may boost the general metabolic performance. Overall, these results suggest a complex interplay between LP09 and LM2. Microcalorimetric data contribute to microbial ecology investigations and related industrial applications in which mixed cultures are utilized, as they unravel the importance of inoculum size, strain

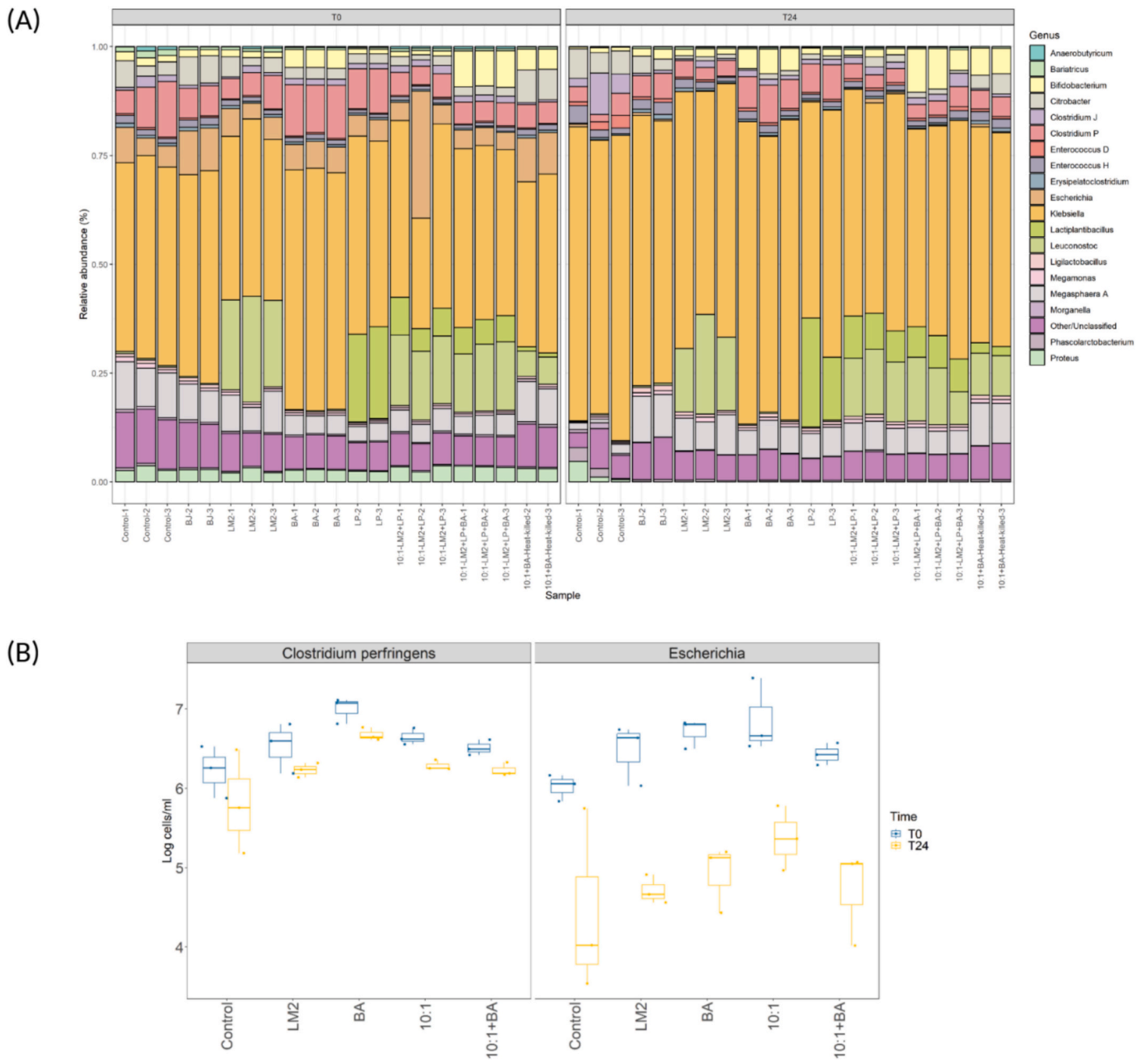


Fig. 5. Ex vivo distal colon model assays to determine the effect of fermented blueberry juice on the gut microbiota. (A) Taxonomic composition of the micro-Matrix samples at 0 h and 24 h expressed in terms of relative abundance at the species level (only taxonomic clades present at >1% relative abundance in at least one sample are shown), and (B) Absolute abundance of taxa showing a significant change at 0 h (blue) and 24 h (yellow) time points in *ex vivo* human distal colon model experiments using the micro-Matrix system in selected samples. Control (FFM), LM2 (FFM + LM2 fermented FBJ), BA (FFM + BJ + BA), 10:1- LM2 + LP (FFM+ LM2 and LP fermented FBJ), 10:1- LM2 + LP + BA (FFM+ LM2 and LP fermented FBJ+ BA). LM2: *L. mesenteroides* VUCC R-037; LP: *Lpb. plantarum* LP09; BA: *Bifidobacterium animalis*. The sample size consisted of three biological samples and one technical replicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dominance, and metabolic timing in optimizing microbial processes. Further investigations are needed to elucidate the precise mechanisms behind these interactions (Gao et al., 2020; Zhou et al., 2015), particularly whether LP09's dominance is due to competitive inhibition, faster substrate utilization, or other factors. Moreover, exploring the metabolic pathways of LM2 in more detail could provide insights into how to optimize its activity in co-culture systems.

The analysis of sugars and organic acids during BJ fermentation revealed distinct strain-specific metabolic behaviours: glucose was broadly consumed by all consortia, while fructose consumption was enhanced in the 10:1 inoculum ratio, supporting the fructophilic adaptation of *L. mesenteroides* LM2. Citric acid remained stable throughout fermentation (between 2.18 and 2.68 g/L), unlike prior reports showing slight declines (He et al., 2024; Wu et al., 2021), possibly due to the limited citric acid metabolism of the strains used. Acetic acid was produced exclusively by LM2 (0.57 g/L) and increased in co-cultures (around 0.7 g/L), contributing with lactic acid to pH reduction, in line with findings by Liao et al. (2023), while the dominant lactic acid production by *Lpb. plantarum* LP09 (2.19 g/L) reflects its faster growth and metabolic capacity, reinforcing the potential of tailored consortia to optimize fermentation profiles.

Antioxidant activity during BJ fermentation revealed that LM2

slightly enhanced DPPH radical scavenging (from 10.1 of blueberry juice to 10.8 μg/mL), while LP09 reduced it (down to 7.4 μg/mL), which is in contrast with Ryu et al. (2019), who observed increased DPPH activity with other *Lpb. plantarum* strains, showing reduced intracellular ROS levels in HepG2 cells. Interestingly, the 10:1 LM2: LP09 ratio improved antioxidant activity compared to LP09 single fermentation (9.1 μg/mL), suggesting that LM2 metabolites may counteract LP09 degrading effects, in line with Hur et al. (Hur et al., 2014), on strain- and substrate-dependent antioxidant responses. FRAP showed minimal impact from LM2, indicating that not all antioxidant compounds were equally affected, likely due to the different antioxidant mechanisms each assay targets (free radical scavenging vs. ferric ion reduction). Finally, the Folin-Ciocalteu assay revealed stable or slightly decreased total phenolic content (between 58 and 53 μg/mL), consistent with the partial utilization or transformation of polyphenols during fermentation potentially increasing their bioavailability as reported by Yang et al. (2023) and Hur et al. (Hur et al., 2014).

The impact of fermentation on the antioxidant properties of blueberry juice was overall modest and likely strain-dependent. The limited variation observed in total phenolic content suggests that fermentation primarily affected antioxidant functionality rather than substantially altering the total phenolic pool. Divergent trends between DPPH and FRAP assays likely reflect their measurement of distinct antioxidant mechanisms, highlighting the complexity of antioxidant responses following fermentation. Accordingly, the interaction between LM2 and LP09 is best interpreted as a strain-dependent modulation of antioxidant properties rather than true synergistic enhancement, with co-culture effects depending on both the metabolic traits of the strains and the analytical approach used.

Overall, among the tested co-culture conditions, the 10:1 LM2:LP09 inoculum ratio showed the most consistent performance across fermentation kinetics and metabolic outputs. This ratio ensured effective acidification and sugar utilization, maintained balanced strain viability, and yielded the most reproducible antioxidant response, and it was therefore selected for subsequent *in vitro* and *ex vivo* analyses.

The cytotoxicity analysis using the MTT assay demonstrated that neither BJ nor FBJ exhibited cytotoxic effects at a 10% treatment concentration after 24 h. These results align with earlier studies (Massarotto et al., 2016; Zhao et al., 2023) which found no adverse effects of blueberries and blueberry extracts on cell viability under comparable conditions. The transepithelial electrical resistance (TEER) measurements indicated that BJ and FBJ can effectively maintain or restore the integrity of tight junctions in cells exposed to lipopolysaccharide (LPS). The restoration of TEER values upon treatment with BJ and FBJ suggests a protective effect which may be attributed to the bioactive compounds present in blueberries. These findings also corroborate those of Marino et al. (2023), who showed that the wild blueberry (*Vaccinium angustifolium*) could similarly enhance TEER values, thereby preserving the function of tight junctions in CaCo-2 cells. The cytochrome *c* assay demonstrated that FBJ possesses considerable antioxidant capacity, both under basal conditions and in the presence of H_2O_2-induced oxidative stress. While the antioxidant activity of FBJ was somewhat lower than that of the commercial BJ, it still showed a marked reduction in oxidative stress compared to the negative control. These findings confirm the well-documented antioxidant properties of BJ, as supported by prior studies (Wang et al., 2017), which demonstrated the superior antioxidant capacity of BJ extract in comparison to grape juice in a cellular antioxidant activity (CAA) assay. Overall data obtained provide valuable insights into the cytoprotective and antioxidant effects of FBJ. The ability of FBJ to protect epithelial barrier integrity and to mitigate oxidative damage further supports its potential as a functional food.

The microMatrix™ distal colon *ex vivo* fermentation system was employed to further corroborate the potential of the FBJ as a functional food, providing a valuable framework to assess its potential impact on the gut microbiota. However, the findings raised several complexities that warrant a careful interpretation, particularly given the observed high abundance of *Klebsiella* spp. (approximately 50%) across all samples, including the control. This notable prevalence suggests that the *ex vivo* distal colon model system was unable to capture the full extent of the gut microbiota in this scenario, as *Klebsiella* spp. is typically not a dominant genus in a healthy gut microbiota. Consequently, while the fermentation process of FBJ and the addition of specific strains (LM2, LP09 as fermentative agents as well as *Bifidobacterium animalis* to evaluate the possible potential of FBJ as a carrier of live probiotics) did allow the detection of those strains in the samples, the overall microbial context is atypical, complicating direct conclusions about FBJ's influence on the gut microbiota *in vivo*.

Despite these limitations, the combination of flow cytometry data and 16S rRNA amplicon sequencing provided useful insights into the absolute abundance of key taxa. A significant observation was the decrease in *Clostridium perfringens* in samples treated with FBJ fermented by both LM2 and LP09 strains, regardless of the addition of *B. animalis* (BA). Similarly, *Escherichia* spp. exhibited a consistent reduction across all FBJ-treated samples. The lack of such changes in the control suggests that FBJ has a potential inhibitory effect on these specific *taxa*, which could be indicative of antimicrobial properties inherent to the BJ itself or the metabolites produced during fermentation. This finding aligns with previous studies that have demonstrated the antimicrobial effects of BJ and its extracts against pathogens such as *Vibrio parahaemolyticus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Yersinia enterocolitica*, and *Staphylococcus aureus* (Lacombe et al., 2012; Sun et al., 2020). While *Escherichia* spp. includes both commensal and pathogenic strains, the reduction of this taxon without affecting the control sample indicates that FBJ may selectively inhibit harmful or opportunistic species within the *Escherichia* genus, which is consistent with earlier reports of blueberry's inhibitory effects on *Escherichia coli* (Lacombe et al., 2012; Sun et al., 2020). This selectivity could be beneficial in modulating gut microbiota balance without broadly disrupting commensal populations.

Although the phenomenon of *Klebsiella* blooms somewhat hampered the precise interpretation of the microbiota results from the above-mentioned *ex vivo* colon model experiments, an optimised Faecal Fermentation Media and experimental conditions were reported (Mukherjee et al., 2025) which significantly mitigated the issue with microbial blooms and consequent loss of microbial diversity. The work reported herein, although conducted with a less optimised media composition that sometimes elicits microbial blooms, remains valid due to its comparability between controls and test samples, enabling us to draw limited, relative and qualitative insights from what is essentially still a pilot lab scale study.

5. Conclusions

This study highlights the potential of blueberry juice fermented with both native (*Leuconostoc mesenteroides* LM2) and probiotic (*Lactiplantibacillus plantarum* LP09) strains to enhance its nutritional, sensory, and health-promoting properties. Strain-specific contributions and complex interactions between LM2 and LP09 optimised fermentation performance, influencing acidification, antioxidant activity, and microbial dynamics, while preserving safety and cellular protective effects. Fermentation trials and associated chemical analysis identified the LM2: LP09 10:1 ratio as the most advantageous since it shows a synergistic production of lactic acid and a predominant contribution of LM2 to acetic acid production, fructose consumption and pH reduction. This ratio was then investigated for key functional analysis such as antioxidant activity, cytotoxicity and gut microbiota modulation. As for the latter, although the *ex vivo* colon model results require cautious interpretation due to atypical microbial communities, the observed selective inhibition of harmful bacteria supports the role of fermented blueberry juice as a promising functional food for gut health and microbial balance. Further investigations, particularly *in vivo* as well as the analysis of individual phenolic compounds, are needed to validate these findings

and elucidate the mechanisms underlying the observed health benefits.

CRediT authorship contribution statement

Nicola Ferremi Leali: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Arghya Mukherjee:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fiona Crispie:** Writing – review & editing, Writing – original draft, Methodology. **Gaston Allendez:** Writing – review & editing, Methodology. **Angela Amoruso:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Romina Monzani:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Serena Allesina:** Writing – review & editing, Methodology, Data curation. **Francesca Deidda:** Methodology, Formal analysis, Data curation. **Daniele Zogno:** Formal analysis, Data curation. **Teresa Graziano:** Writing – review & editing, Methodology. **Marco Corazzari:** Writing – review & editing, Supervision, Formal analysis. **Paul D. Cotter:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Marco Pane:** Writing – review & editing, Supervision, Conceptualization. **Harsh Mathur:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation. **Sandra Torriani:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Elisa Salvetti:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Data curation.

Ethics

Ethics approval for this study was granted by the Clinical Research Ethics Committee (CREC) in Cork, Ireland for study number APC179. This approved study only involved the collection of faecal samples for the purposes of human distal colon model experiments described herein.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.118696.

Data availability

Raw 16S rRNA amplicon sequencing data generated in this study was submitted to the European Nucleotide Archive under BioProject PRJEB96788 (secondary accession: ERP179402).

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