Sustainable Food Technology

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
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Cite this: *Sustainable Food Technol.*, 2024, **2**, 1101

Use of whey for a sustainable production of postbiotics with potential bioactive metabolites

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Postbiotics have gained attention due to their health benefits and potential bioactive metabolites. Short-chain fatty acids (SCFAs) have been identified within these metabolites, which are related to anti-inflammatory properties and antioxidant activity, among others. For the food industry, it is important to consider a suitable culture medium for postbiotic production. Whey, as a by-product from the cheese industry, is rich in nutrients and is proposed to support this purpose. This study is aimed to evaluate the microbial growth of three probiotics, *Lactiplantibacillus plantarum* 299v, *Lacticaseibacillus casei* Shirota, and *Bifidobacterium animalis* subsp. *lactis* BPL1, using a whey culture medium supplemented with soluble fibres (inulin or chia mucilage) at two concentrations (1% or 2% w/w). Also, analyse the effect of soluble fibres on the production of SCFAs and the antioxidant activity of cell-free supernatant as postbiotics. SCFA production was quantified by HPLC and antioxidant activity was determined by the $DPPH^+$ assay and the $KMnO_4$ agar method. The formulated culture media promoted the growth of probiotics, especially those added with inulin. *Lactiplantibacillus plantarum* 299v and *Lacticaseibacillus casei* Shirota produced primary lactic and acetic acid. *B. lactis* BPL1 had the highest SCFAs production in the culture medium with 2% w/w of inulin. The antioxidant activity from *Lactiplantibacillus plantarum* 299v postbiotics was significantly improved with soluble fibres ($p < 0.05$). This study shows postbiotics are produced with a sustainable approach. Moreover, postbiotics based on whey and soluble fibres can be a potential ingredient for the formulation of new food products as sources of SCFAs and antioxidants.

Received 20th February 2024
Accepted 11th June 2024

DOI: 10.1039/d4fb00061g

rsc.li/susfoodtech

Sustainability spotlight

The sustainable production of postbiotics, rich in antioxidants and short-chain fatty acids (SCFAs), using whey and soluble fibres in culture media is an approach that promotes the principles of the circular economy through the effective use of by-products of the dairy industry such as whey. The use of renewable resources through soluble plant-based fibers, reduces overall environmental impact, offering health benefits to consumers, optimizes nutrient utilization and aligns with market growth demand for sustainable and functional food products.

1 Introduction

Postbiotic is a relatively new term in industry, food science, and technology research. A wide variety of concepts describe postbiotics; the most current one was given by the consensus panel of the International Scientific Association of Probiotics and Prebiotics (ISAPP). They defined postbiotics as the "preparation of inanimate microorganisms and/or their components that confer a benefit to the health of the host".[1] Postbiotics can provide potential functional properties such as antimicrobial, antioxidant, and strengthen the immune system, which can positively affect microbiota homeostasis and/or consumer metabolic and signaling pathways.[2] Incorporating postbiotics into foods has been proposed since they have certain advantages compared to probiotics, such as longer shelf life, storage, handling, and easier transportation.[3] The cell-free supernatants (CFS) are considered postbiotics that contain many bioactive metabolites besides the culture media. Short-chain fatty acids (SCFAs) are of great interest among these metabolites for their possible health benefits.[4] The main SCFAs produced during bacterial fermentation of soluble fibres are acetate, butyrate, and propionate. SCFAs can confer certain effects such as anti-tumor, anti-inflammatory in the colon, cardiovascular, obesity control, control of glucose homeostasis, appetite regulation, and protection against the development of immune disorders.[5] Also, the consumption of SCFAs as postbiotics through diet has been proposed.[6] Several studies focused on food metabolomics have proven that the fermentation of probiotics (*Lactobacillus* and *Bifidobacterium* species) with soluble fibres can produce SCFAs.[7–10]

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On the other hand, in recent years, postbiotics' antioxidant activity has gained relevance in food science research. Oxidative stress can cause an imbalance of biological processes at the cellular level, such as stem cell depletion, tumorigenesis, autoimmunity (immune response against autoantigens), and accelerated senescence.[11] It has been demonstrated that postbiotics (*Lactobacillus plantarum* AF1 and *L. brevis* KCCM 12203P) have interesting antioxidant activities.[12,13] Some researchers[14] have mentioned that conventional techniques (ABTS and DDPH assay) have certain complications in effectively measuring antioxidant activity. For this reason, the method based on potassium permanganate ($KMnO_4$), which is simple, fast to develop, reliable, and economically feasible, has been proposed.

For postbiotics production on an industrial scale, it is essential to have a culture medium suitable for the microorganisms' growth and the production of bioactive metabolites, and preferably, be economically feasible and safe for consumption. Generally, the culture mediums used are laboratory formulations (MRS broth) or milk; however, on a large scale, these can be expensive. Whey has been reported to be suitable for the growth of probiotics,[15] since it is rich in sugars (lactose 46–52 g L^{-1}), protein (6.5–6.6 g L^{-1}), and minerals (5.0–5.2 g L^{-1}).[16] Besides, whey is a by-product of the cheese industry, is relatively cheap, and is easily accessible. Still, it is sometimes discarded and unused as it should be.[17] The cheese industry is known to produce an estimated 115 million tons of whey annually, of which 47% of the by-product is directly disposed of in drains. This is a matter of deep concern, leading to severe environmental pollution issues such as water contamination, dissolved oxygen depletion, and eutrophication.[16] Whey has a high biochemical oxygen demand (BOD) ranging from 40–60 g L^{-1} and chemical oxygen demand (COD) ranging from 50–80 g L^{-1}. These high levels of BOD and COD make whey a significant pollutant by-product worldwide, posing a significant environmental challenge for dairy industries. Furthermore, few studies currently focus on evaluating alternative culture mediums for producing postbiotics that are suitable for the food industry.[3,18,19] Recently, Yousefi *et al.*[20] optimised the fermentation conditions of *L. plantarum* in whey to improve the antibacterial metabolites production whereas Izzo *et al.*[21] analysed the antifungal activity and phenolic compounds from the fermented goat's sweet whey utilized *L. plantarum* strains. Amiri *et al.*[18] optimised the supplementation of cheese whey to improve the production of conjugated linoleic acid, exopolysaccharides, and bacteriocins by *B. lactis* BB12 as postbiotics. It is evident the increasing interest to utilise whey as culture medium to produce functional metabolites with food applications. Moreover, it has been reported that several soluble fibers rich in glucose, arabinose, galactose, fructose, fructooligosaccharides (FOS), galactomannans, among others, promotes postbiotic production.[22,23]

Therefore, this research aims to evaluate the microbial growth of probiotics (*Lactiplantibacillus plantarum* 299v, *Lacticaseibacillus casei* Shirota, and *Bifidobacterium animalis* subsp. *lactis* BPL1) using a whey culture medium supplemented with soluble fibres (inulin or chia mucilage) at two concentrations (1% or 2% w/w). Also, analyse the production of SCFAs and the antioxidant activity of cell-free supernatant as postbiotics.

2 Material and methods

2.1 Probiotic strains

To obtain postbiotics, it is essential to consider heterofermentative probiotic bacteria to ensure the production of SCFAs. *Lactiplantibacillus plantarum* 299v, *Lacticaseibacillus casei* Shirota, and *Bifidobacterium animalis* subsp. *lactis* BPL1 are well known probiotics.[24–28] Thus, these strains were selected to produce postbiotics. The probiotic strains *Lactiplantibacillus plantarum* 299v and *Bifidobacterium animalis* subsp. *lactis* BPL1 were obtained in the freeze-dried form of Digestive probiotic (Nature Made, West Hills, CA) and Microbiot® Fit (Grupo Columbia, México City, Mexico), and *Lacticaseibacillus casei* Shirota was obtained from the Food Microbiology Laboratory of the Universidad de las Americas Puebla. *Lactiplantibacillus plantarum* 299v and *L. casei* Shirota were reactivated and subcultivated in MRS broth (Difco™, BD, Sparks, MD, USA) at 35 °C. For the reactivation and sub-cultivation of *B. lactis* BPL1, MRS broth was supplemented with 0.5% (w/v) cysteine hydrochloride (Sigma-Aldrich, St. Louis, MO) and grown under anaerobic conditions at 35 °C.

2.2 Chia mucilage extraction

The chia seeds (*Salvia hispanica* L.) were obtained from Verde Limón Trading Company (Mexico City, Mexico). For the extraction of mucilage, chia seeds were hydrated in distilled water at a ratio of 1 : 20 (w/v) for 4 h with constant stirring; then, seeds were mechanically separated using a strainer mesh #35 (500 μm), and the mucilage was lyophilized (Triad™ Labconco, USA).[29]

2.3 Preparation of culture medium

For the culture medium, the following ingredients were used: whey powder (Food Technologies Trading, Mexico), yeast extract (Difco™, BD, Sparks, MD, USA), magnesium sulfate ($MgSO_4 \cdot 7H_2O$) (Merck, Burlington, MA), manganese sulfate ($MnSO_4 \cdot H_2O$) (Merck, Burlington, MA), inulin (Agaviotica, Jalisco, Mexico) and chia mucilage. The culture medium used as control (CW) was composed of whey powder 10% w/w, yeast extract 0.3% w/w, $MgSO_4 \cdot 7H_2O$ 0.02% w/w, $MnSO_4 \cdot 4H_2O$ 0.005% w/w, and water. Based on previous experiments, it was decided to use a range of soluble fibres concentrations of 1% and 2% w/w. Then, 4 culture media were formulated to evaluate inulin and chia mucilage to enrich the CW: CW plus inulin 1% w/w (WIn1%), CW plus inulin 2% w/w (WIn2%), CW plus chia mucilage 1% w/w (WMc1%) and CW plus chia mucilage 2% w/w (WMc2%). The culture medium for *B. lactis* BPL1 was supplemented with vitamins C (Sigma-Aldrich, St. Louis, MO) and E (Sigma-Aldrich, St. Louis, MO) at 250 ppm and 5.75 ppm, respectively, to provide adequate reducing conditions for bacteria growth. All culture media were sterilized at 120 ± 1.0 °C for 15 min and stored at 25 °C until used. Since MRS broth has been used as a culture medium in most of the studies with

postbiotics;[30] it was used as reference to compare the culture mediums formulated in this study.

2.4 Probiotic culture conditions and bacterial kinetic growth

Fresh cultures of the probiotics (MRS broth) were used to inoculate CW media, adding the necessary amount to obtain an initial population of 10^6 CFU mL^{-1}. The culture mediums were incubated without stirring at 35 °C for 30 h. For the bacterial growth curve, samples were taken from culture media at 0, 3, 6, 9, 24, and 30 h. For microbial counting, MRS agar (Difco™, BD, Sparks, MD, USA) was used by the surface extension method. After appropriate incubation, colonies were counted. The kinetics of bacterial growth were modelled using the Modified Gompertz equation (eqn (1)). Where A is the maximum net growth, μ is the exponential growth rate (h^{-1}) and λ is the latency time (h). These kinetic parameters were obtained by nonlinear regression. Minitab 20 software (Minitab LLC, State College, PA, USA) was used to calculate the residual analysis (RMSE) and correlation coefficients (R^2). The kinetics of bacterial growth were carried out by triplicate.

$$\log\frac{N}{N_0} = A \times \exp\left[-\exp\left[\left(\frac{\mu \times \exp(1)}{A}\right)(\lambda - t) + 1\right]\right] \quad (1)$$

2.5 pH and titratable acidity

Samples were taken from the culture medium to analyse pH and titratable acidity (TA) during fermentation (0, 9, 24, and 30 h). pH was determined by immersion electrode using a pH meter (HI 2210 Hanna Instruments, Woonsocket, RI, USA). The TA was determined following the method 22.061 from AOAC[31] and was expressed as a percentage of lactic acid (% w/v). The measurements were performed in triplicate.

2.6 Cell-free supernatant preparation as postbiotics

At the end of fermentation, samples were centrifuged at 7000*g* for 15 min at 5 °C (Sorvall ST 8R, Thermo Fischer Scientific, Schwerte, Germany). The supernatant was then filtered by 0.45 μm cellulose nitrate filter (Advantec, MFS, Dublin, CA, USA). Finally, the cell-free supernatants were frozen at −18 °C until their use.

2.7 Characterization of postbiotics

2.7.1 Determination of lactic acid and short-chain fatty acids (SCFAs). To analyse the production of organic acids in the different media, the concentration of lactic, acetic, propionic, and butyric acid was determined and quantified by performing high-performance liquid chromatography (HPLC) based on the methodology reported by Hernández-Figueroa *et al.*[32] The chromatograph used was an Agilent 1260 (Agilent Technologies, Santa Clara, CA, USA) coupled with a diode array detector (DAD) at a wavelength of 210 nm. CFS was injected (20 μL) with an Agilent G1329 autosampler (Agilent Technologies, Santa Clara, CA, USA). An Aminex HPX-87H column (300 × 7.8 mm) (BIO-RAD, Hercules, CA, USA) was used with a mobile isocratic phase of 20 mM monobasic potassium phosphate buffer solution (adjusted-pH at 2.4) to 0.6 mL min^{-1} at room temperature. For the quantification of lactic acid and SCFAs, standard solutions of 12.5 to 200 mM of lactic, acetic, propionic, and butyric acids were prepared. The concentration of acids was linearly correlated with their respective peak area, obtaining correlation coefficients $R^2 > 0.99$. This analysis was performed by duplicate for every postbiotic.

2.7.2 Total phenolic content of postbiotics. The total phenolic content was determined employing a Folin–Ciocalteu reaction following the methodology of Seo *et al.*[33] Fifty microliters of CFS were mixed with 50 μL of 5% Folin–Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) with a time of incubation of 5 min at room temperature in dark. Posteriorly, 100 μL of 20% Na_2CO_3 solution were added and incubated at room temperature for 20 min. Then, the reaction product was measured at 730 nm employing a spectrophotometer Multiskan Skyhigh reader (ThermoFisher Scientific, MS, USA). The total phenolic content was expressed in mg of gallic acid (GAE) per L from the standard curve (500–50 mg GAE per L) with $R^2 > 0.95$. The determination of every postbiotic was carried out in triplicate.

2.8 Antioxidant activity of postbiotics

2.8.1 $KMNO_4$ agar method. The total antioxidant activity (TAA) from the CFS was determined following the method of Hanchi *et al.*[14] Briefly, $KMnO_4$ agar was prepared from a 0.5 mM solution of $KMnO_4$ (Sigma-Aldrich, St. Louis, MO) with 1.5% bacteriological agar (Bioxon, BD, Mexico City, Mexico). For the agar plates, 20 mL of $KMnO_4$–agar was poured into 60 mm Petri dishes. Uniformed wells of 7.4 mm were made to add the CFS to the agar plates. Subsequently, $KMNO_4$ agar plates were stored in darkness for 24 h at 4 °C. In addition, a solution of 5.5 mM of ascorbic acid (Sigma-Aldrich, St. Louis, MO) was prepared and tested as a conventional antioxidant agent. The antioxidant activity was calculated with eqn (2), where TAA is the total antioxidant activity (mm), D_b is the diameter of the bleached or colorless area and D_c is the negative control diameter (unfermented culture media or distilled water). The test was carried out in triplicate.

$$\mathrm{TAA} = D_b - D_c \quad (2)$$

2.8.2 DDPH radical scavenging activity of postbiotics. For this determination, the methodology of Xing *et al.*[34] was used. Briefly, 100 μL of 0.2 mM DDPH (CAS 1898-66-4, Sigma Aldrich, MO, USA) solution were mixed with 100 μL of postbiotic sample. Then, the mixture was incubated for 30 min in dark conditions at room temperature. The samples with DDPH were measured at 517 nm using Multiskan Skyhigh reader (ThermoFisher Scientific, MS, USA). The scavenging activity was calculated with eqn (3), where A_b is the absorbance of the blank and A_s is the absorbance of the sample. The assay was carried out by triplicate.

$$\text{Scavenging activity } \% = (A_b - A_s)/A_b \times 100 \quad (3)$$

2.9 Statistical analysis

The data were analysed using Analysis of Variance (ANOVA) and Tukey's mean comparison test, with a confidence level of 95%. Minitab 20 software (Minitab LLC, State College, PA, USA) was utilized for the analysis.

3 Results and discussion

3.1 Probiotics growth in the culture media

The metabolites such as SCFAs are produced by different metabolic pathways of fermentation during the microbial growth. MRS broth used as the reference culture medium was optimal for all evaluated probiotics (Fig. 1). Considering that metabolites' production occurs primordialy during the exponential phase of the microorganisms kinetics, we can highlight that the value of the exponential phase growth rate μ for *Lactiplantibacillus plantarum* 299v was 0.50 h^{-1} with MRS medium, but with no significant difference ($p > 0.05$) when using the whey formulations supplemented with chia mucilage or inulin (Table 1). Even though, a clear maximum microbial net growth (A parameter of 3.50 log CFU ml^{-1}) was obtained for *L. plantarum* 299v with MRS broth (Fig. 1, Table 1). For *Lacticaseibacillus casei* Shirota and *Bifidobacterium animalis* subsp. *lactis* BPL1, the highest μ value was attained with the WIn1% formulated medium (0.66 and 0.48 h^{-1}), with no significant difference when using MRS broth ($p > 0.05$). Burns *et al.*[15] studied the suitability of whey for the growth of probiotics (*L. acidophilus* A9, 08, and H5; *L. paracasei* A13 and LS; and *L. casei* LB) showing that whey medium supplemented with yeast extract (like CW) was optimal for probiotic growth. However, the maximum net growth values (A) reported by Burns *et al.*[15] were lower (1.4–1.6 log N/N_0) than those obtained in the present study. In another study, Hernandez-Mendoza *et al.*[35] modelled the growth of *L. reuteri* NRRL 1417, and *B. bifidum* NCFB2715 in a whey-based food beverage with the modified Gompertz equation, and the A values were also lower (1.76–0.97 log N/N_0) than our results.

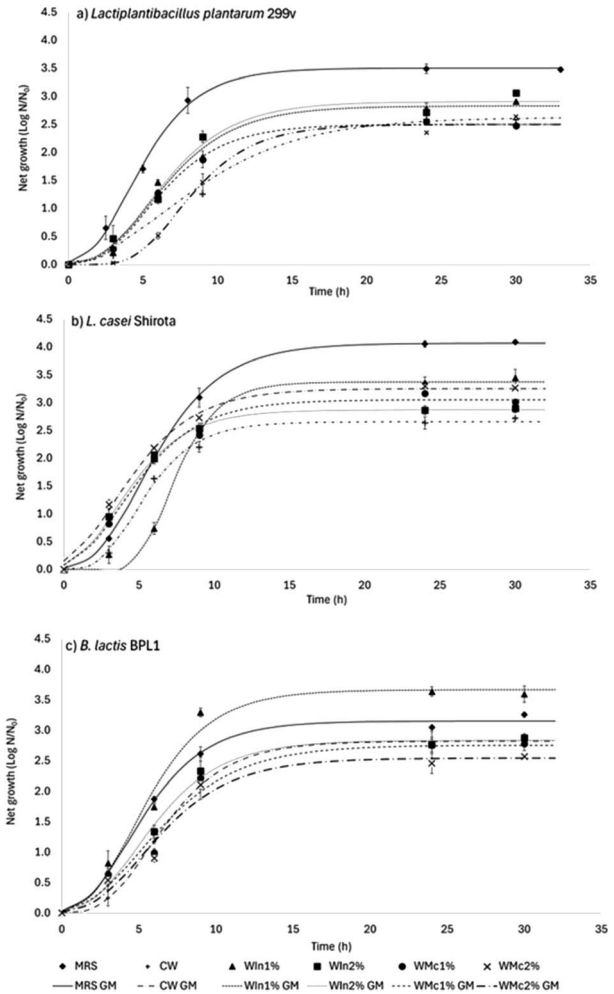

Fig. 1 Probiotic bacteria growth (symbols) and prediction (lines) of modified Gompertz (GM) equation of *Lactiplantibacillus plantarum* 299v, *Lacticaseibacillus casei* Shirota, and *Bifidobacterium lactis* BPL1 in the culture mediums. MRS: de Man, Rogosa and Sharpe broth; CW: control; WIn1%: CW plus 1% w/w inulin; WIn2%: CW plus 2% w/w inulin; WMc1%: CW plus 1% w/w chia mucilage; WMc2%: CW plus 2% w/w chia mucilage.

It is well known that inulin is a good prebiotic and enhances the probiotics' growth.[36–39] The probiotics studied adapted better to the medium supplemented with inulin than those with chia mucilage. The A values of WIn1% (*Lacticaseibacillus casei* Shirota and *B. lactis* BPL1) and WIn2% (*Lactiplantibacillus plantarum* 299v) were significantly higher ($p < 0.05$) than the WMc1% and WMc2% medium. In previous investigations with *B. lactis* BPL1 have reported the stimulation of *Bifidobacterium* species in culture media containing inulin.[37,38] However, the concentration of inulin should be evaluated since high concentrations may cause adverse effects on *Bifidobacterium* growth. McLaughlin *et al.*[36] reported that several species of *Bifidobacterium* did not grow adequately at high concentrations (>5% w/v) of inulin. Similar behaviour was observed in the present study with *B. lactis* BPL1, which showed a reduction in the growth (parameters μ and A) with 2% of inulin. This growth reduction is attributed to a saturation of substrate due to bacteria inability to metabolize all the soluble fibre.[36] Likewise, *Lacticaseibacillus casei* Shirota had the highest μ_m value at the lowest inulin concentration (1% w/w), showing that this probiotic was better adapted to soluble fibre. This finding was similar to the research of Renye *et al.*,[39] in which several strains of *Lacticaseibacillus casei* presented greater growth when the medium was supplemented with inulin. In contrast, the chia mucilage had no significant effect ($p > 0.05$) on the growth rate, probably due to chia mucilage being a very complex carbohydrate, composed primarily of rhamnogalacturonan and arabinoxylans, that probiotics do not metabolize.[40] To the best of our knowledge, no previous reports were found about chia mucilage fermentation. The μ value is important since the fermentation time is reduced at higher rates, which could benefit large-scale production costs. Furthermore, a rapid exponential growth rate is key for generating metabolites of interest, such as SCFAs. These components are considered secondary metabolites,

Table 1 Kinetic parameters of the modified Gompertz equation describing the growth of probiotic strains in several culture mediums[a]

Medium	A ($\log N/N_0$, CFU mL^{-1})	μ (h^{-1})	λ (h)	R^2	RSME
***Lactiplantibacillus plantarum* 299v**					
MRS	3.50 ± 0.06^{A}	0.50 ± 0.03^{A}	1.42 ± 0.23^{B}	0.996	0.125
CW	2.60 ± 0.08^{BC}	0.21 ± 0.03^{B}	1.14 ± 0.05^{C}	0.988	0.158
WIn1%	2.82 ± 0.07^{BC}	0.36 ± 0.04^{AB}	1.60 ± 0.39^{B}	0.993	0.133
WIn2%	2.90 ± 0.07^{B}	0.38 ± 0.04^{AB}	1.68 ± 0.36^{B}	0.990	0.166
WMc1%	2.50 ± 0.05^{C}	0.35 ± 0.03^{AB}	1.66 ± 0.30^{B}	0.995	0.104
WMc2%	2.50 ± 0.04^{C}	0.34 ± 0.03^{AB}	3.54 ± 0.30^{A}	0.996	0.098
***Lacticaseibacillus casei* Shirota**					
MRS	4.06 ± 0.04^{A}	0.50 ± 0.02^{A}	1.99 ± 0.15^{BC}	0.999	0.078
CW	2.65 ± 0.04^{E}	0.43 ± 0.03^{B}	2.35 ± 0.19^{B}	0.997	0.085
WIn1%	3.37 ± 0.07^{B}	0.66 ± 0.06^{A}	4.91 ± 0.24^{A}	0.995	0.153
WIn2%	2.87 ± 0.04^{DE}	0.41 ± 0.02^{B}	0.75 ± 0.21^{D}	0.997	0.086
WMc1%	3.05 ± 0.05^{CD}	0.39 ± 0.03^{B}	0.83 ± 0.29^{CD}	0.995	0.120
WMc2%	3.25 ± 0.04^{BC}	0.41 ± 0.02^{B}	0.35 ± 0.21^{D}	0.997	0.093
***Bifidobacterium animalis* subsp. *lactis* BPL1**					
MRS	3.15 ± 0.04^{B}	0.42 ± 0.02^{A}	1.47 ± 0.18^{A}	0.998	0.080
CW	2.82 ± 0.05^{BC}	0.37 ± 0.03^{B}	2.76 ± 0.28^{A}	0.996	0.103
WIn1%	3.67 ± 0.09^{A}	0.48 ± 0.05^{A}	1.70 ± 0.40^{A}	0.990	0.206
WIn2%	2.84 ± 0.07^{BC}	0.35 ± 0.03^{B}	1.79 ± 0.39^{A}	0.991	0.147
WMc1%	2.80 ± 0.08^{BC}	0.29 ± 0.04^{B}	1.61 ± 0.56^{A}	0.986	0.183
WMc2%	2.55 ± 0.08^{C}	0.29 ± 0.04^{B}	1.93 ± 0.58^{A}	0.983	0.183

[a] MRS: de Man, Rogosa and Sharpe broth; CW: control; WIn1%: CW plus 1% w/w inulin; WIn2%: CW plus 2% w/w inulin; WMc1%: CW plus 1% w/w chia mucilage; WMc2%: CW plus 2% w/w chia mucilage. Different letters show a significant difference ($p < 0.05$) between the culture mediums of each probiotic.

presumed to be synthesized at the beginning and during the stationary phase.[41]

The latency time (λ) represents the bacteria's adaptation time in the medium to grow due to changes in culture conditions. *Lactiplantibacillus plantarum* 299v required 3.54 h to start growing in the culture medium supplemented with WMc2%, being the longest observed time ($p < 0.05$). This result can be due to the high viscosity of the culture medium with chia mucilage causing an obstacle to the probiotic adaptation in the culture medium since the nutrients are inaccessible to the microorganisms. *B. lactis* BPL1 adapted rapidly to all tested culture mediums; showing a λ value very similar ($p > 0.05$).

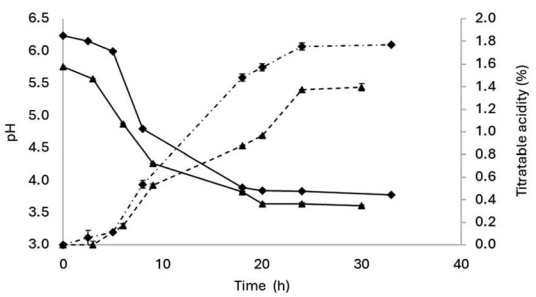

Fig. 2 Evolution of pH and titratable acidity in de Man, Rogosa, and Sharpe broth (MRS) (◆) and control medium (CW) (■) for *Lactiplantibacillus plantarum* 299v.

3.2 pH and TA of culture media during the growth

Lactiplantibacillus plantarum 299v presented a drastic reduction in pH and an increase in TA from 9 to 16 h of fermentation (Fig. 2); this behaviour was similar for all culture medium and probiotics. For *Lacticaseibacillus casei* Shirota, the pH values after 30 h of fermentation in CW was 3.55 and did not show significant differences ($p > 0.05$) when supplementing with chia mucilage or 2% of inulin (Fig. 3). It results important to highlight that *B. lactis* BPL1 presented the lowest pH (3.3 ± 0.01) values in WIn1% ($p < 0.05$) among all culture media and compared with the other evaluated lactobacilli. These data were similar to those reported by several studies that used whey as a growth medium.[15,42,43] The TA after 30 h of fermentation in the culture medium ranged from 2.04 to 1.05% (see Fig. 3). There were significant differences ($p < 0.05$) in TA values of the three probiotics in MRS broth compared with the studied culture mediums. Regarding the whey-based mediums supplemented with soluble fibers, *Lacticaseibacillus casei* Shirota showed the highest values of TA in WIn2%, while for *Lactiplantibacillus plantarum* 299v was with WMc1% and WMc2%. For *Lactiplantibacillus plantarum* 299v, TA increased directly proportional to inulin concentration; this effect agrees with previous reports with *L. plantarum* species.[44,45] It is important to emphasize that the pH and TA values of the culture mediums at the end of fermentation were more acidic than those reported in the literature. For instance, a study of the fermentation of a whey-based beverage using *L. reuteri* NRRL 14171 and *B. bifidum* NCFB2715 recorded pH values of 4.50–4.86 and TA of 0.315–0.378%.[35] The difference in pH and TA between the

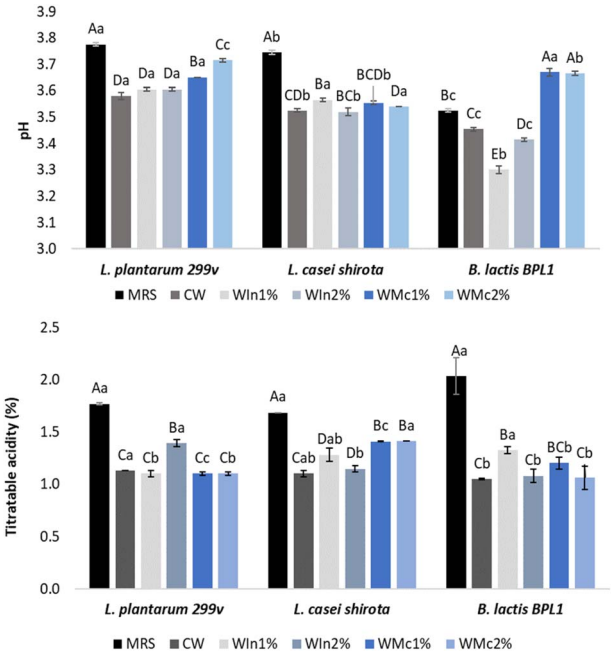

Fig. 3 pH and titratable acidity values of culture media fermented with *Lactiplantibacillus plantarum* 299v, *Lacticaseibacillus casei* Shirota and *Bifidobacterium animalis* subsp. *lactis* BPL1 after 30 h at 37 °C. TA is expressed as lactic acid (% w/v). Different capital letters indicate significant differences between culture medium ($p < 0.05$). Different lower-case letters indicate significant differences ($p < 0.05$) between probiotics in the same medium. MRS: de Man, Rogosa and Sharpe broth; CW: control; WIn1%: CW plus 1% w/w inulin; WIn2%: CW plus 2% w/w inulin; WMc1%: CW plus 1% w/w chia mucilage; WMc2%: CW plus 2% w/w chia mucilage.

previous study and this work may be due to the fermentation time.

3.3 Analysis of lactic acid and short-chain fatty acids postbiotics

As is observed in Table 2, postbiotics of *Lactiplantibacillus plantarum* 299v or *B. lactis* BPL1 from MRS broth had the highest ($p < 0.05$) concentration of lactic acid. *Lacticaseibacillus casei* Shirota yielded the highest amount ($p < 0.05$) of lactic acid from WIn1% in the tested culture medium. Furthermore, a significant effect ($p < 0.05$) was observed in all postbiotics from a whey-based medium supplemented with inulin, increasing the concentration of lactic acid. Despite previous reports mentioning that some strains of *L. plantarum* are not capable of degrading inulin enzymatically, the hydrolysis of inulin in acidic conditions (pH $\leq$ 4.0) occurs, releasing simple monomers of fructose and glucose, which bacteria could use as carbon source.[45–48] Also, Matusek *et al.*[49] observed that oligosaccharide degradation increased with pH reduction. They also mentioned that several strains of *Lacticaseibacillus casei* can ferment FOS and inulin.

Concerning the SCFAs, the acetic acid was reported with the highest concentration ($p < 0.05$) among all postbiotics from MRS broth. In the whey-based media, the supplementation of chia mucilage increased significantly ($p < 0.05$) the concentration of acetic acid by *Lactiplantibacillus plantarum* 299v postbiotics. Tamargo *et al.*[50] assessed the effect of chia mucilage on the microbiota using a dynamic gastrointestinal model (Simgi®). The addition of 0.8% chia mucilage stimulated the SCFAs production but did not specified which microorganisms were the producers. Similar to this study, they observed that acetic acid was the primary fermentation product of chia mucilage samples analysed with Simgi®. Probably, acetic acid production was favoured by the major chia mucilage's sugars such as xylose, glucose, arabinose, galactose, glucuronic acid, and galacturonic acid.[22] Xylose, arabinose and compounds derived from galactomannan are important precursors in acetic acid production by the glycolytic pathway (*Lactobacilli*) and pentose phosphate (*Bifidobacterium*).[23] Inulin significantly ($p < 0.05$) increased the concentration of acetic acid in the postbiotics of *Lacticaseibacillus casei* Shirota or *B. lactis* BPL1. The lactic and acetic acid concentrations of culture medium based on whey were similar to those reported by Renye *et al.*[39] (190 and 18 mM, respectively).

As expected, *Lactobacilli* and *Bifidobacterium* produced different organic acids. The presence of propionic and butyric acid was only detected in the postbiotics of *B. lactis* BPL1, in which the WIn2% postbiotics had the highest amounts ($p < 0.05$). This difference in the production of SCFAs is because *Lactobacilli* uses the glycolytic pathway (forming pyruvate) and phosphoketolase pathway under heterofermentative conditions. *Bifidobacterium* species produce organic acids *via* the pentose phosphate pathway using the enzyme fructose-6-phosphate phosphoketolase (F6PPK).[51] In addition, the fermentation of FOS is given by the hydrolytic enzyme and β-fructofuranosidase, which has been related to *B. adolescentis*, *B. breve*, and *B. animalis* subsp. *lactis*.[36] Similarly, Renye *et al.*[39] investigated the production of SCFAs in MRS broth and inulin from various probiotics. *B. breve* 2141 produced ≈4.5 mM of propionic acid and ≈1.0 mM of butyric acid. In the same way, Ozcan & Eroglu[52] employed MRS broth with 1% inulin to grow *L. acidophilus* and analyse the SFCAs production. Their results showed minimal propionic acid concentrations (1.34 mM) and butyric acid (0.87 mM).

Studies focused on the metabolomics of fermentations with probiotics have identified a great diversity of metabolites, of which SCFAs have stood out.[53] A metabolomic profile study from the fermentation of whey with *L. plantarum* MTCC 5690 only detected the presence of one SCFA (butyric acid).[54] Therefore, the supplementation of soluble fibers is necessary to increase these compounds. This was demonstrated with a metabolomic study carried out by Tay *et al.*,[55] who observed that adding soluble fiber (okara) increased the concentration of SCFAs in postbiotics from *Bifidobacterium* spp. It is important to recognize that *Bifidobacterium* in lactic fermentations are great probiotic producers of SCFAs.[56]

As could be noted, *B. lactis* BPL1 was the largest producer of propionic and butyric acid with WIn2% medium. Propionate has health benefits such as obesity control, glucose homeostasis control, appetite regulation, and potential cardiovascular effects, due to propionate can activate G-Protein Coupled Receptor 41 (GPCR41) which stimulates the satiety hormone

Table 2 Organic acids concentration at 30 h of fermentation of each probiotic strain in the culture media[a]

Medium	Lactic acid (mM)	Acetic acid (mM)	Butyric acid (mM)	Propionic acid (mM)
***Lactiplantibacillus plantarum* 299v**				
MRS	247.53 ± 0.85^{A}	42.98 ± 1.29^{A}	ND	ND
CW	93.06 ± 0.49^{E}	18.91 ± 1.39^{C}	ND	ND
WIn1%	142.32 ± 5.22^{C}	18.53 ± 0.48^{C}	ND	ND
WIn2%	193.08 ± 1.15^{B}	12.54 ± 0.43^{D}	ND	ND
WMc1%	104.49 ± 0.85^{D}	28.28 ± 0.11^{B}	ND	ND
WMc2%	94.56 ± 3.41^{DE}	27.86 ± 0.79^{B}	ND	ND
***Lacticaseibacillus casei* Shirota**				
MRS	160.82 ± 1.16^{B}	75.09 ± 1.78^{A}	ND	ND
CW	145.63 ± 1.46^{DE}	14.68 ± 0.28^{C}	ND	ND
WIn1%	170.86 ± 0.43^{A}	18.42 ± 0.06^{B}	ND	ND
WIn2%	154.42 ± 1.65^{BC}	17.72 ± 0.09^{B}	ND	ND
WMc1%	151.46 ± 1.14^{CD}	16.98 ± 0.14^{BC}	ND	ND
WMc2%	138.72 ± 3.48^{E}	15.63 ± 0.43^{BC}	ND	ND
***Bifidobacterium animalis* subsp. *lactis* BPL1**				
MRS	152.93 ± 2.06^{A}	69.65 ± 1.16^{A}	9.79 ± 0.58^{C}	4.71 ± 0.05^{C}
CW	94.37 ± 1.35^{E}	13.01 ± 0.22^{E}	14.62 ± 0.21^{AB}	4.58 ± 0.02^{C}
WIn1%	143.5 ± 1.8^{B}	28.41 ± 0.36^{B}	13.5 ± 0.09^{B}	17.09 ± 0.23^{B}
WIn2%	97.56 ± 2.52^{E}	17.35 ± 0.23^{D}	16.07 ± 0.96^{A}	18.58 ± 0.39^{A}
WMc1%	106.89 ± 0.98^{D}	25.61 ± 0.54^{C}	ND	ND
WMc2%	117.46 ± 0.77^{C}	23.43 ± 0.21^{C}	ND	ND

[a] ND: no detectable; MRS: de Man, Rogosa and Sharpe broth; CW: control whey; WIn1%: whey plus 1% w/w inulin; WIn2%: whey plus 2% w/w inulin; WMc1%: whey plus 1% w/w chia mucilage; WMc2%: whey plus 2% w/w chia mucilage. Different letters show a significant difference ($P < 0.05$) between the culture mediums of each probiotic.

leptin *via* adipocytes.[5] Butyrate can be used as an energy source for epithelial cells and has been studied for its antitumor effects. The mechanism of action has not yet been fully understood. In addition, it has been suggested that butyrate inhibits histone deacetylases that dominate the intracellular gene expression of cells, preventing cancer cell proliferation.[5,57]

In respect to functional concentrations of SCFAs, Nakkarach *et al.*[58] studied the postbiotic anticancer and anti-inflammatory effects from *Escherichia coli* KUB-36. The SCFAs concentrations tested were low (21.28 mM acetic acid, 0.50 mM propionic acid, and 0.47 mM butyric acid). The SCFAs influenced the inhibition of several cancer cell lines (MCF10-A, MCF7, HT-29 and leukemia cancer cells) and inhibited the expression of proinflammatory cytokines (IL1β, IL6 and TNF-α). Hence, the concentrations reported in the present study with *B. lactis* BPL1 could have a similar or greater anti-inflammatory and anticancer activity.

3.4 Total phenolic content of postbiotics

Phenolic compounds from LAB have been shown to play an important role in antioxidant capacity[34,59,60] The total phenolic content of postbiotics is shown in Fig. 4. All postbiotics obtained from the MRS broth showed the highest phenolic content ($p < 0.05$) compared to the formulated mediums using whey (449.97–378.90 mg GAE per L). Regarding *B. lactis* BPL1, CW total phenolic content (396.82 mg GAE per L) from CW did not show significant differences ($p > 0.05$) compared to MRS. The mechanism of action of phenolic compounds as antioxidants is mainly given by their capability to donate hydrogen to free radicals avoiding oxidative chain reactions such as lipid oxidation.[34] In addition, it has been reported that *Lactiplantibacillus plantarum* species can produce phenolic acids such as DL-3-phenyllactic acid, salicylic acid, and vanillin, and their production varies according to the culture medium used.[61] Other study reported gallic acid, protocatechuic acid, chlorogenic acid, syringic acid, vanillin acid, *p*-coumaric acid, 4-

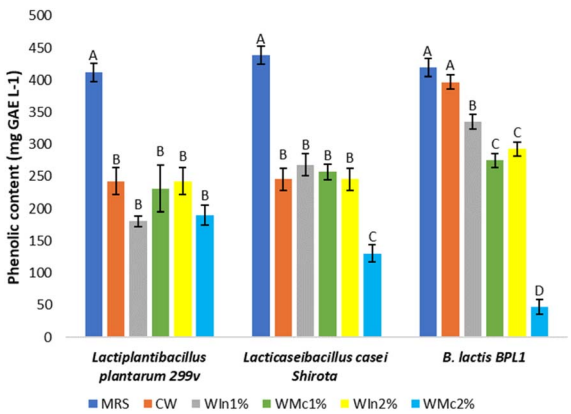

Fig. 4 Phenolic content of postbiotics from Man, Rogosa and Sharpe broth (MRS), control whey medium (CW), whey plus 1% w/w inulin (WIn1%) and whey plus 1% w/w chia mucilage (WMc1%), whey plus 2% w/w inulin (WIn2%), whey plus 2% w/w chia mucilage (WMc2%) of *Lactiplantibacillus plantarum* 299v, *Lacticaseibacillus casei* Shirota and *Bifidobacterium animalis* subsp. *lactis* BPL1. Different letters show a significant difference ($p < 0.05$) between the culture mediums of each probiotic.

hydroxybenzoic acid, hydrocinnamic acid, sinapic acid, DL-3-phenyllactic acid (PLA), and 1,2 dihydroxybenzene as the main phenolic compounds produced by four *L. plantarum* strains in goat's sweet whey after 72 h; PLA was the predominant phenolic compound.[21] İncili *et al.*[59] have mentioned that phenolic compounds (mainly phenolic acids) are not only antioxidant compounds but also antimicrobials.

The importance of phenolic compounds in the human diet focuses on flavonoids which can be used by microbiota in the large intestine as carbon source to produce SCFA providing beneficial physiological benefits to the host (mitigation of obesity, regulates glucose metabolism, inhibitor of lipid synthesis, cancer preventive effects, among others).[62] Thus, the formulated postbiotics provide SCFA and their precursors at the same time, which can supply potential health benefits. Also, few studies suggest selected flavonoids suppress the growth of pathogens like *Clostridium perfrigens*, *Clostridium difficile*, *Bacteroides*, *Escherichia coli*, and *Salmonella* leading to potential microbiome's modulation.[63] Moreover, flavonoids are recognised as antioxidants and to act as a direct radical scavenging with health benefits such as anticancer and anti-inflammatory, among others.[63–65]

3.5 Antioxidant activity of postbiotics

Only postbiotics from media supplemented with 1% soluble fibres (WIn1% and WMc1%), whey-based (CW), and MRS broth were considered for this determination. Fig. 5a shows the diameters of antioxidant activity of the postbiotics, and Fig. 6 shows the colourless diameters in plates of $KMnO_4$ agar method. The MRS postbiotics had the largest ($p < 0.05$) diameter of antioxidant activity among the studied postbiotics, which was similar ($p > 0.05$) to the ascorbic acid solution. The antioxidant activity from *Lactiplantibacillus plantarum* 299v postbiotics was significantly improved with soluble fibres ($p < 0.05$). The diameters of antioxidant activity from *Lacticaseibacillus casei* Shirota postbiotics were not modified by the soluble fibres ($p > 0.05$). For *B. lactis* BPL1 postbiotics, the chia mucilage (1%) decreased the antioxidant activity ($p < 0.05$) compared with inulin or CW. Likewise, the postbiotics from *Lactiplantibacillus plantarum* 299v and *L. rhamnosus* GG in MRS presented similar antioxidant activity (diameters of 10 to 15 mm) compared to *Lacticaseibacillus casei* Shirota of this study.[14] On the other hand, the postbiotics' scavenging activity measures using DDPH had a similar tendency (Fig. 5b). For instance, adding soluble fibres such as inulin and chia mucilage in *Lactiplantibacillus plantarum* 299v growth media significantly increased ($p < 0.05$) the inhibition of DDPH similar to the $KMnO_4$ agar method. It is reported that probiotics fermentation generates antioxidant compounds such as glutathione (GSH), butyrate, folate, lactate, 3-phenyllactate, indole-3-lactate, β-hydroxybutyrate, γ-aminobutyrate and flavonoids.[21,66,67] In the present study, the presence of butyrate in the postbiotics of *B. lactis* BPL1 was demonstrated, and this compound has been associated with antioxidant activity in previous studies.[68] Flavonoids (as phenolic compounds) from postbiotics as mentioned in section 3.4 provide antioxidant and direct radical scavenging

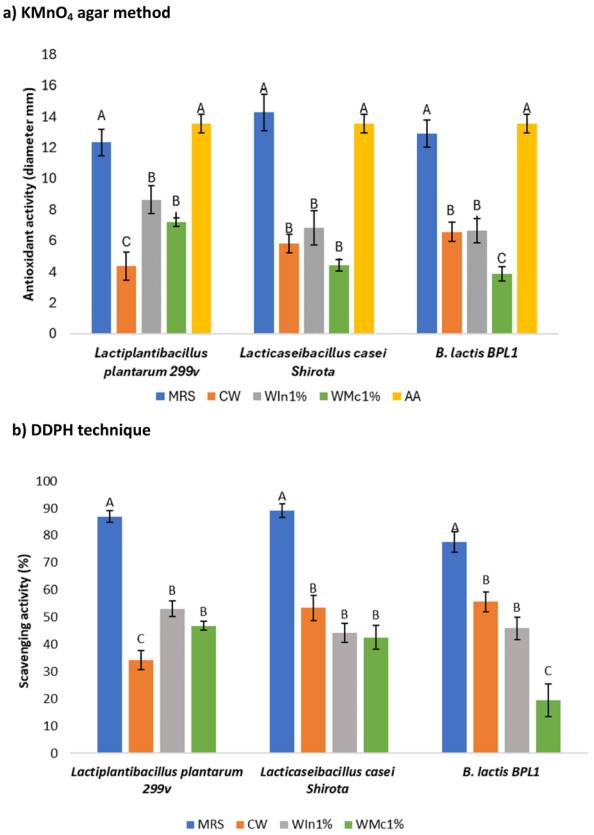

Fig. 5 Antioxidant activity by (a) $KMNO_4$ agar method and (b) DDPH technique of postbiotics from de Man, Rogosa and Sharpe broth (MRS), control whey medium (CW), whey plus 1% w/w inulin (WIn1%) and whey plus 1% w/w chia mucilage (WMc1%) of *Lactiplantibacillus plantarum* 299v, *Lacticaseibacillus casei* Shirota and *Bifidobacterium animalis* subsp. *lactis* BPL1. Different capital letter indicates significant differences between ($p < 0.05$) culture media.

activity potentially with anti-cancer and anti-inflammatory action. However, the identification and specific analyses of flavonoids in the postbiotics are necessary. In addition, whey proteins have antioxidative potential as scavenger of free radicals (sulfhydryl containing amino acids) and chelating transition metal ions (lactoferrin) which enhance after whey fermentation.[54,69] To show the effect of soluble fibers in the antioxidant activity, the activity from the culture medium with unfermented soluble fibers (control) was subtracted from the obtained data. The diameters in the $KMNO_4$ agar method and inhibition of DDPH were negligible with the unfermented medium. Furthermore, the antioxidant activity of soluble fibers was reviewed in the literature, and at similar concentrations to the tested inulin, the antioxidant activity is minimal.[70]

Even though the DDPH method is the most common to analyse the antioxidant activity, their use in postbiotics is difficult due to sample provides turbidity. The $KMnO_4$ agar method was convenient, and there was no interference in the measurement of colourless diameters, although the results took longer time (24 h) than the DDPH technique. Therefore, the $KMnO_4$ agar method demonstrated to be a viable option for

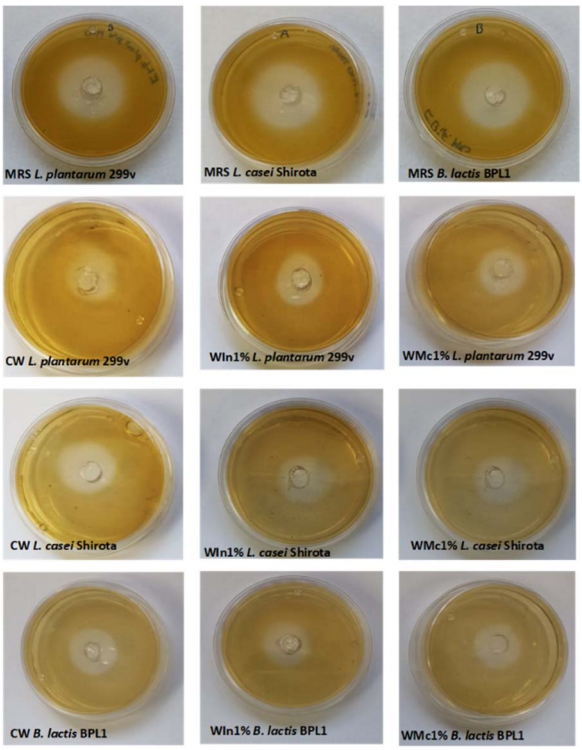

Fig. 6 Antioxidant activity (colourless diameters) of postbiotics from Man, Rogosa and Sharpe broth (MRS), control whey medium (CW), whey plus 1% w/w inulin (WIn1%) and whey plus 1% w/w chia mucilage (WMc1%) of *Lactiplantibacillus plantarum* 299v, *Lacticaseibacillus casei* Shirota and *Bifidobacterium lactis* BPL1.

determining the antioxidant capacity of postbiotics as Hanchi *et al.*[14] validated the method of antioxidant activity.

4 Conclusions

The whey-based culture media supplemented with inulin (WIn1%-WIn2%) were optimal for microbial growth of *Lactiplantibacillus plantarum* 299v, *Lacticaseibacillus casei* Shirota and *B. lactis* BPL1. The organic acids in postbiotics, *Lactiplantibacillus plantarum* 299v and *Lacticaseibacillus casei* Shirota were primary lactic and acetic acid, while *B. lactis* BPL1 showed lactic, acetic, propionic, and butyric acid. The supplementation of soluble fibres improved antioxidant activity in postbiotics of *Lactiplantibacillus plantarum* 299v. In summary, the whey-based culture mediums supplemented with inulin are suitable for microbial growth, SCFAs production, and antioxidant activity sources. Therefore, the postbiotics from these culture mediums could be an economical and sustainable option for human consumption to incorporate postbiotics with SCFAs and antioxidant activity in developing novel food products. The use of whey in the production of postbiotics not only contributes to the effective utilization of byproducts from the cheese industry, avoiding critical environmental issues, but also aligns with sustainability principles, resource efficiency and health promotion. Incorporating these postbiotics into new food products further extends sustainable practices into the consumer market. In future research, it would be interesting to explore the metabolomics of studied probiotics growing in whey and soluble fibres. Finally, investigate the safety, stability, sensory, and bioactive effects of the postbiotics in food products.

Author contributions

Conceptualization, V. E. V.-S., M. T. J.-M., E. M.-L. and A. L.-M.; visualization: V. E. V.-S.; validation, V. E. V.-S., M. T. J.-M., E. M.-L. and A. L.-M.; investigation, V. E. V.-S.; resources, M. T. J.-M. and A. L.-M.; writing—original draft preparation, V. E. V.-S.; writing—review and editing, V. E. V.-S., M. T. J.-M., E. M.-L. and A. L.-M. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The author Vera-Santander appreciates the financial support for his doctoral studies in Food Science from the National Council of Humanities, Sciences and Technologies (CONAHCyT) and Universidad de las Américas Puebla (UDLAP).

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