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High-performance anion-exchange chromatography coupled with pulsed amperometric detection and capillary zone electrophoresis with indirect ultra violet detection as powerful tools to evaluate prebiotic properties of fructooligosaccharides and inulin

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Abstract

Fructooligosaccharides (FOS) and inulin are food grade non-digestible carbohydrates that exert beneficial nutritional effect. This paper describes the suitability of high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) and capillary zone electrophoresis (CZE) to evaluate fermentation properties of FOS and inulin in pure *Bifidobacterium* cultures; and to study their effects on faecal cultures (microbial population and short-chain fatty acids). Prebiotic effectiveness of FOS and inulin of different degrees of polymerization was evaluated monitoring the changes in their molecular weight distribution during the in vitro growth of selected *Bifidobacterium* strains. The qualitative analysis of the residual soluble oligosaccharides or polysaccharides from Raftilose Synergy, Raftiline HP and Raftilose P95 was carried out by HPAEC-PAD, using a CarboPac PA100 column and an appositely optimized gradient elution program. Under the optimized gradient elution conditions, glucose, fructose, sucrose were resolved from each other and from fructans with a DP ranging from 3 (1-kestose) to 60. The chromatographic profiles of the spent broths pointed out that almost every strain presented a different capability to ferment fructan chains of variable DP, indicating wide strain to strain differences. To explore the prebiotic effect of FOS and inulin, related to of short chain fatty acids (SCFAs) accumulation in faecal cultures due to fermentative metabolism of intestinal microflora, analysis of SCFAs, acetic and lactic acid was achieved by co-electroosmotic capillary electrophoresis, where the electrophoretic mobility of the anionic analytes and electroosmotic flow (EOF) were similarly directed. Moreover, the use of UV detection for the analyses of our organic anions required a running electrolyte which allowed indirect detection. The optimization of the capillary electrophoretic conditions was carried out by applying a chemometric study based on the use of the experimental design, the effects of three parameters, i.e. temperature, voltage and percentage of methanol added to the background electrolyte were investigated.

Keywords: Fructooligosaccharides; Inulin

1. Introduction

Functional foods provide a new way of expressing healthiness in food choices. A food, as well as a food ingredient, can be regarded as functional "if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects" [1].

Interesting food ingredients in this respect are fructooligosaccharides (FOS) and inulin, water-soluble carbohydrates which are distinguished in fructans groups. Fructans are carbohydrate polymers consisting of a sucrose molecule that is elongated by a chain of fructosyl units connected

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through β-(2 → 1) or β-(2 → 6) linkages [2], depending on the linkage type they are called inulin and levan, respectively.

Levans are highly branched fructose polymers with β-(2 → 6) linkages for the main chain and β-(2 → 1) branching, whereas inulin is a linear, highly polymerized fructan of degree of polymerization (DP) 11–60, produced by extraction from chicory roots, and consisting of a linear chain of fructose with β-(2 → 1) linkages with a terminal glucose unit.

Fructooligosaccharides with DP 3–10 (average DP 4.5) are produced during the process of chemical degradation or controlled enzymatic hydrolysis of inulin by endoglycosidases [3–4]. Furthermore, FOS can be produced on a commercial scale, from sucrose, using a fungal enzyme from either *Aureobasidium* sp. [5] or *Aspergillus niger* [6].

Among functional foods, prebiotics are non-digestible ingredients that beneficially affect the human health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. FOS and inulin are not degraded or adsorbed in the stomach or in the small intestine and reach the colon intact; here they promote the proliferation of bifidobacteria, which constitute a significant portion of the intestinal microflora and are claimed to have several beneficial effects on the host. For the perceived benefits on human health [7–9], FOS and inulin are identified as prebiotics and are probably the most commonly used prebiotic fibers in the production of functional foods.

High-performance anion-exchange chromatographic (HPAEC), coupled with pulsed amperometric detection (PAD), enables complete, single step separation of neutral and charged oligo and polysaccharides differing by branch, linkage, and positional isomerism [10]. One of the aims of the present work was the specific optimization of this technique to evaluate the fructan fermentation patterns of bifidobacteria, by the qualitative variations of oligo and/or polysaccharide compositions of either FOS or inulin.

Beneficial effects of prebiotics are mediated by short chain fatty acids (SCFAs), the outcome of carbohydrate microbial fermentation in the colon [11]. In order to characterize more deeply FOS and inulin as prebiotics, an innovative method to analyze SCFAs in faecal cultures, based on capillary electrophoresis, was developed. Capillary electrophoresis (CE) with indirect UV detection is a valuable detection method for non-UV-absorbing low-molecular-mass ions and reversing the electroosmotic flow (EOF) is essential to achieve rapid CE separations of anionic analytes. The direction of the EOF can be reversed by a chemical modification of the capillary wall [12–13] or by dynamic coatings adding suitable electrolyte additives [14–16]. Alternatively, a promising approach is the use of a polycationic electroosmotic modifier such as hexadimethrine bromide (HDB) [17–18]. In our approach, the dynamic coating of the bare fused-silica capillary with hexadimethrine bromide was performed prior the electrophoretic separations which were conducted using mixtures of benzoic acid–methanol as the running electrolyte.

In this paper, results regarding HPAEC-PAD analysis of fructans of bifidobacteria cultural broths, as well as CZE determinations of their effect on short chain fatty acid production are presented and discussed.

2. Material and methods

2.1. Samples and chemicals

Raftilose Synergy, Raftiline HP, and Raftilose P95 were supplied by Orafti (Tienen, Belgium). They are mixtures of chicory FOS and inulin containing selected DP distribution. In Raftilose Synergy, FOS represent the largest portion, but it is present also a fraction of long chain inulin; Raftiline HP consists mostly of long chain inulin (average DP = 25, absent FOS with DP <5); Raftilose P95 is composed by 95% FOS (DP 3–10). All short chain fatty acids, benzoic acid, tris[hydroxymethyl]aminomethane, hexadimethrine bromide (HDB) and 5-hydroxymethyl-2-furaldehyde were purchased from Sigma–Aldrich (Steinheim, Germany). All standard reagents were of analytical grade. Fifty percent (w/w) sodium hydroxide solution, sodium acetate anhydrous and HPLC-grade methanol were purchased from J.T. Baker (Deventer, The Netherlands). All sample solutions were made with HPLC-grade water and filtered through a Type 0.22 μm single-use membrane filter (Millipore, Bedford, MA, USA).

2.2. Instrumentation

Carbohydrate analyses were performed with a Dionex (Sunnyvale, CA, USA) Model 4000i gradient pump module equipped with a pulsed electrochemical detector (PED) consisting of an amperometric flow through cell with a gold working electrode and a silver–silver chloride reference electrode. The time and voltage parameters on the PED detector in the pulsed amperometric mode were set as follows: E_1, E_2 and E_3 were, respectively, +0.10, +0.60, and −0.60 V, with the assigned pulsed durations t_1 0–0.50 s; t_2 0.51–0.59 s; t_3 0.60–0.65 s. Integration of the signal occurred between 0.30 and 0.50 s. Samples were injected using a Rheodyne Model 9125 non-metal (peek) injection valve with a peek sample loop of 10 μL (Cotati, CA, USA). Separations were performed at room temperature on a Dionex CarboPac PA100 column, connected to the associated guard column. Chromatographic data were collected and plotted using the Dionex AI-450 chromatography workstation.

Gradient elution was applied using three solvents: water (eluent A), 0.6 M aqueous sodium hydroxide (eluent B) and 0.5 M aqueous sodium acetate solution (eluent C). Total run time per sample was 110 min at a flow-rate of 0.8 mL min^{-1}. The elution began with 89% eluent A, 10% eluent B and 1% eluent C for 5 min. This was followed by a first linear gradient

step from 10 to 20% eluent B and from 1 to 30% eluent C in elent A for 50 min, followed by a second linear gradient step from 20 to 25% eluent B and from 30 to 75% elunt C for 50 min and maintained at these conditions for 5 min. The column was then washed for 10 min with 100% eluent B. The next injection was performed after equilibrating the column with 89% eluent A, 10% eluent B and 1% eluent C for 20 min. All mobile phases were sparged and pressurised with helium to prevent adsorption of atmospheric carbon dioxide and subsequent production of carbonate, which would act as displacing ion and shorten retention times.

All capillary electrophoretic experiments were performed on a P/ACE MDQ system (Beckman-Coulter, Fullerton, CA, USA), with UV detection at 214 nm. Separations were carried out in an untreated fused-silica capillary (Quadrex, New Haven, CT, USA) of 75 μm i.d. and an effective length of 50 cm (total length 60 cm).

All samples were hydrodynamically injected into the capillary in 5 s at 0.5 psi (1 psi = 6.9 kPa), and an applied voltage of −20 kV, unless otherwise indicated. All voltage used herein were experimentally verified to be within the linear region of the Ohm's plots. The measurements were run at least in triplicate to ensure reproducibility. All electrophoresis runs were performed at temperature 30 °C. The on-column detection window was made by burning a small section (ca. 3 mm) of the external polyimide coating and scraping off the burned residue with methanol. The separation steps were done automatically and controlled by Beckman P/ACE MDQ system version 2.2 software (Beckman-Coulter).

2.3. Dynamic coating of the inner wall of fused silica capillary

Prior the first use, the untreated capillary was treated by rinsing at high pressure (20 psi) with 0.5 M NaOH for 20 min and water for 5 min and then with 0.1 M hydrochloric acid (20 min), followed by a second treatment with water (5 min), sodium hydroxide (0.1 M for 20 min), water (5 min). The capillary was dynamically coated with a solution of 0.2% (w/v) of hexadimethrine in water, which was flushed at high pressure (20 psi) for 5 min.

The dynamic coating was re-applied using the above procedure after each nine electrophoretic runs conduced with a background electrolyte consisting of 24% (v/v) of methanol in a solution of 15 mM sodium benzoate with the pH adjusted by adding 1.0 M Tris–base buffer to 6.22. After each run the capillary was flushed with the background electrolyte for 3 min.

2.4. Solutions and sample preparation

Individual stock solutions of each analyte were prepared prior to use at a concentration of 20–250 mM in purified water and stored at −20 °C. Adequate aliquots were diluted in order to generate the work calibration standards. Supernatant samples from batch fermentations and faecal cultures were appropriately diluted and filtered prior either HPAE-PAD or CZE analyses.

2.5. Strains, culture conditions and growth evaluation

Bifidobacterium strains used in this study were obtained from ATCC (Rockville, MD, USA), DSMZ (Braunschweig, Germany) and University of Bologna (Italy) culture collections.

Cells from MRS (Difco) stock cultures were inoculated into a minimal synthetic medium with composition ($g\,L^{-1}$): Raftilose Synergy or Raftiline HP, 20; Casaminoacids (Difco), 15; Yeast Nitrogen Base (Difco), 6.7; ascorbic acid, 10; sodium acetate, 10; $(NH_4)_2SO_4$, 5; urea, 2; $MgSO_4{\cdot}7H_2O$, 0.2; $FeSO_4{\cdot}7H_2O$, 0.01; $MnSO_4{\cdot}7H_2O$, 0.007; NaCl, 0.01; Tween 80, 1; cysteine, 0.5; pH adjusted to 7.0. The carbohydrate was autoclaved separately and added to the sterile basal medium to give the required concentration.

Batch fermentations of bifidobacteria were performed on Raftilose Synergy or Raftiline HP as sole carbon sources. Cultures were incubated at 37 °C for 48 h. Samples were centrifuged (10,000 × *g*, 10 min) and supernatants were filtered through a 0.22 μm membrane and directly injected into the HPAEC-PAD for detection of residual oligo and polysaccharides.

Growth of pure *Bifidobacterium* cultures on Raftilose Synergy and Raftiline as sole carbon source was monitored by increasing of turbidity, measured as optical density at 600 nm (OD_{600}), and lowering of pH, as result of production of lactic and acetic acids from carbohydrate fermentation. Growth was considered satatistically significant ($P < 0.05$) on the basis of paired samples Student's *t*-test between pH and turbidity values before and after 48 h of fermentation.

2.6. Faecal cultures

The 0.4 mL of Wilkins-Chalgren Anaerobe Broth (Oxoid) 1% (v/v) faecal suspensions were used to inoculate anaerobic serum bottles containing 40 mL of the following medium ($g\,L^{-1}$): yeast extract, 5; sodium acetate, 1; $(NH_4)_2SO_4$, 5; urea, 2; $MgSO_4{\cdot}7H_2O$, 0.2; $FeSO_4{\cdot}7H_2O$, 0.01; $MnSO_4{\cdot}7H_2O$, 0.007; NaCl, 0.01; Tween 80, 1; hemin 0.05; cysteine, 0.5; Raftilose P95 or Raftiline HP as test carbohydrates, 10; pH 7.0.

Faecal cultures were incubated at 37 °C for 24 h and supernatants were periodically collected for HPAEC-PAD determination of residual fructans and CE analysis of SCFAs. Bifidobacteria were enumerated soon after inoculation and after 24 h of fermentation by fluorescent in situ hybridization technique (FISH) using the commercial kit Bifidobacterium 10-ME-H001 (Microscreen B.V., Microbial Diagnostics, Groeningen, The Netherlands). The slides were observed with a Nikon Eclipse E-600 epifluorescence microscope equipped with a mercury arc lamp (Nikon, HBO, 100 W) and FITC specific filter (Nikon, BA 520).

3. Results and discussion

3.1. Optimization of HPAEC-PAD for fructan detection in microbial cultures

With the purpose to find the best elution gradient conditions that comprise good resolution at the beginning as well as at the end of a chromatographic run and elution of as long fructans as possible, various eluent combinations were tested to enable the selective elution with high reproducible retention time of FOS and inulin with degree of polymerization (DP) up to 60. Baseline separation in a single run, was obtained using the gradient profile described in the Section 2. The separation was designed to be completed within 110 min. Using the mild start conditions of the optimized gradient profile, glucose, fructose, sucrose were resolved from each other and from fructans with a DP ranging from 3 (1-kestose) to 60, achieving the selective separation of both low molecular weight and high molecular weight fructan mixtures in the whole molecular weight range. The assignment of the chromatographic peaks with DP higher than 3 was based on the generally accepted assumptions that the retention time of a homologous series of carbohydrates increased as the DP increased, and that each successive peak represented a fructan which had a fructose more than that of the previous peak. Moreover, the individual peaks were sharp and well resolved, strongly suggesting that FOS and fructans were, as expected, linear.

Furthermore, the high sensitivity and specificity of integrated pulsed amperometric detection for the analyzed carbohydrates minimized possible interferences and simplified sample preparation. Chromatograms regarding oligo and polysaccharide distribution in both Raftilose Synergy and Raftiline HP are depicted in Fig. 1. By the comparison of the chromatographic profiles is evinced that an abundant oligosaccharide fraction is present in Raftilose Synergy, whereas fructans at higher DP are singularly present in a smaller amount, but with a qualitative DP distribution similar as in Raftiline HP in which oligosaccharides are practically absent.

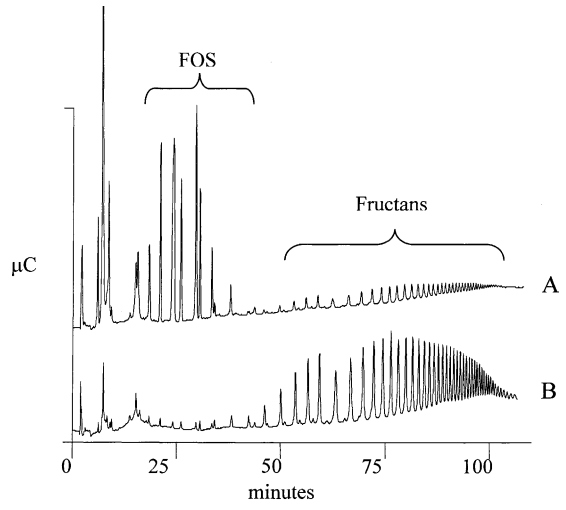

Fig. 1. Chromatographic profiles of Raftilose Synergy (A) and Riftiline HP (B) obtained by HPAEC-PAD under elution conditions reported in Section 2.2.

Over the course of the chromatographic elution, the relative standard deviations of the retention times of all eluted peaks (optimized conditions, $n = 6$) ranged from 0.20 to 1.25%.

3.2. HPAEC-PAD characterization of residual FOS and inulin in Bifidobacterium and faecal cultures

In the present study, HPAEC-PAD technique was exploited for monitoring the fructan consumption in *Bifidobacterium* and faecal cultures growing on FOS or inulin as sole carbon sources.

The degradation of FOS and inulin was determined for *Bifidobacterium* strains that grew during 48 h of incubation in a minimal synthetic medium supplemented of 10 g L^{-1} Raftilose Synergy or Raftiline HP.

HPAEC-PAD chromatographic profiles allowed the comparison of the strains respect to their fermentation capabilities of FOS or inulin, indicating wide strain to strain differences. For all strains a clear correlation of degradation pattern with growth was observed, i.e. the strains showing the lowest final pH and the highest A_{600} presented the highest degree of degradation.

As examples, Fig. 2 reports the chromatographic profiles of Raftilose Synergy before (A) and after incubation with *Bifidobacterium cuniculi* MB280 (B) and *Bifidobacterium adolescentis* ATCC15703 (C), giving the final pH and OD_{600} values of 6.12, 0.40 and 4.87, 1.11, respectively. Fig. 3 reports the chromatographic profiles of Raftiline HP before (A) and after incubation with *Bifidobacterium cuniculi* MB280 (B) and *Bifidobacterium adolescentis* ATCC15703 (C), giving the final pH and OD_{600} values of 5.02, 0.57 and 5.13, 1.74, respectively.

In order to evaluate the fructans consumption performed by mixed faecal cultures, strictly anaerobic fermentation vessels were inoculated with faecal samples. They contained all the nutrients that could support growth of intestinal bacteria and FOS (Raftilose P95) or inulin (Raftiline HP) as carbon sources.

HPAEC-PAD analysis suggested that the fermentation of FOS and inulin by faecal population resulted always in the complete consumption of these carbohydrates. Mean concentration of bifidobacteria, enumerated by FISH soon after inoculation and after 24 h of incubation at 37 °C, in all analyzed samples is increased from their baseline level 1.9×10^5 to 3.7×10^7 and 1.2×10^7 on FOS and inulin, respectively. Typical chromatographic profiles obtained in fermentation with FOS (Raftilose P95) and inulin (Raftiline HP) as sole carbon sources are depicted in Figs. 4 and 5, respectively.

The microbiological study [19] comparing the growth of 55 *Bifidobacterium* strains on FOS and inulin demonstrated that all grew abundantly on glucose and FOS, whereas only

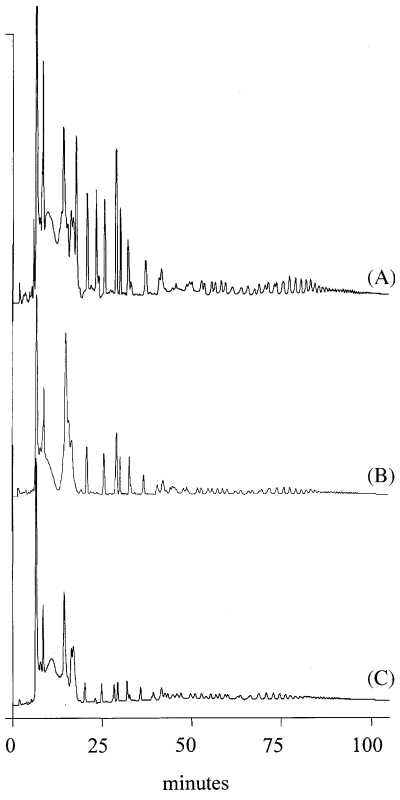

Fig. 2. Chromatograms of Raftilose Synergy before (A) and after incubation with *Bifidobacterium cuniculi* MB280 (B) and *Bifidobacterium adolescentis* ATCC15703 (C). Chromatographic conditions as in Fig. 1.

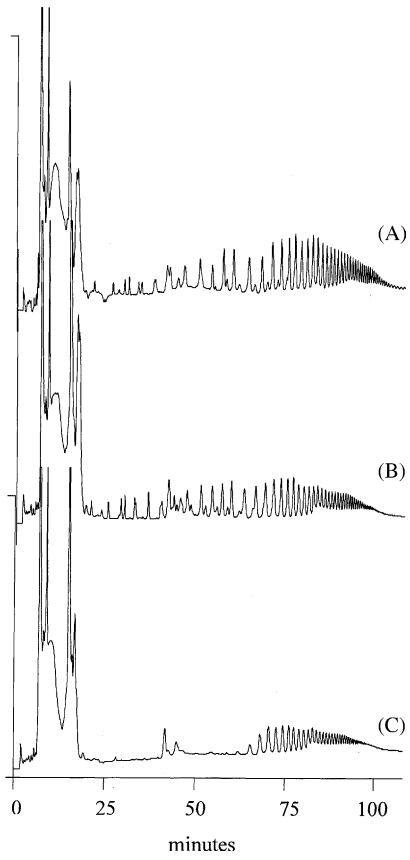

Fig. 3. Chromatograms of Raftiline HP before (A) and after incubation with *Bifidobacterium cuniculi* MB280 (B) and *Bifidobacterium adolescentis* ATCC 15703 (C). Chromatographic conditions as in Fig. 1.

eight were able to grow on inulin. Nevertheless, in faecal cultures, up to the longest chains of inulin were completely consumed by the heterogeneous intestinal microflora.

These results suggest that HPAEC-PAD is a powerful tool for analysis of oligo- and polysaccharides in complex mixtures such as microbial and faecal cultures and can provide significant information about interactions among bacteria and prebiotic fibers.

3.3. CZE separation of acetate, lactate and SCFAs

To obtain information on the nature and the quantity of acetic, lactic acid and short-chain fatty acids in faecal cultures, we developed a capillary electrophoretic separation method adopting a co-electroosmotic flow (co-EOF) approach. Manipulation of the EOF is essential for achieving optimized rapid separations of small anions by CZE. Extremely rapid separation of organic and inorganic anions can be achieved by CE if the electrophoretic mobility and the EOF are in the same direction (co-EOF). The most common method to reverse the direction of the EOF, is the addition of a cationic surfactant to the background electrolyte. In this work reversal of EOF was achieved using as dynamic coating agent hexadimethrine (HDM) bromide, a polycationic polymer which is absorbed to the capillary surface due to the strong electrostatic attraction between the polymer and the

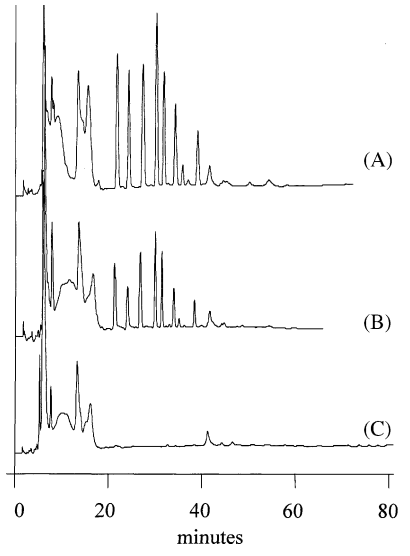

Fig. 4. Chromatographic profiles obtained during fermentation of Raftilose P95 by a faecal culture. Profile (A) at the beginning of fermentation; (B) after 9 h; (C) after 24 h. Chromatographic conditions as Fig. l.

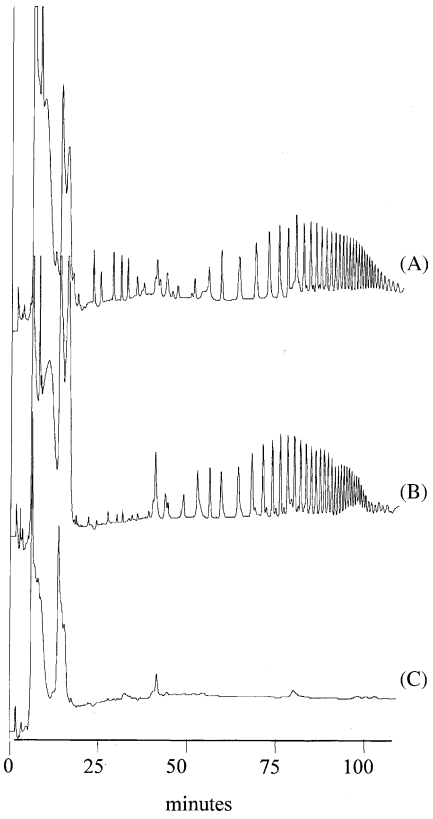

Fig. 5. Chromatographic profiles obtained during fermentation of Raftiline HP by a faecal culture. Profile (A) at the beginning of fermentation; (B) after 9 h; (C) after 24 h. Chromatographic conditions as Fig. 1.

silanols on the capillary wall, thus reversing the sign of the zeta potential [20]. In this way, we were able to obtain a stable capillary coating for EOF reversal without the addition of a EOF modifier in the running electrolyte, which can be undesiderable if micelles are formed and interact with analyte anions or using electrospray mass spectrometry detection [21].

In order to verify the dynamic coating stability with HDM, a mixture of nitrate and nitrite was used as a test solute. Applying a negative voltage (−25 kV) and using 20 mM phosphate buffer at pH 2.50 as the running electrolyte, the negatively charged nitrate and nitrite eluted before 5-HMF, which was selected as a EOF marker. Migration time, of both analytes and their observed and effective mobility were chosen as a test to verify their repeatability between runs and between different dynamic coatings. The obtained results of nine consecutive electrophoretic runs are summarized in Table 1. The EOF never decreased by more than 1.75% over the stability test. Day-to-day reproducibility of the EOF measurements was less than 2.5%, repeating the dynamic coating for 10 days.

The use of UV detection for the analysis of the non-absorbing acetate, lactate and SCFAs anions required a running electrolyte which allowed indirect detection. For the indirect UV detection the background electrolyte consisted of 24% (v/v) of methanol in a solution of 15 mM sodium benzoate with the pH adjusted by adding 1.0 M Tris–base buffer to 6.22. The addition of an organic solvent such as methanol to the running electrolyte was effective for decreasing EOF and to improve selectivity regarding some anions such as resolution between lactic acid–propionic acid and butyric acid-2-hydroxyvaleric acid.

Table 1
Repeatability of migration time (t_m), apparent (μ_{obs}) and effective mobility (μ_{eff}) of dynamic coated capillary with hexadimethrine

	Mean value[a]	S.D.	R.S.D. (%)
Nitrate			
t_m (min)	1.68	0.004	0.26
μ_{obs} ($cm^2\ V^{-1}\ S^{-1}$)	11.31×10^{-4}	2.96×10^{-6}	0.26
μ_{eff} ($cm^2\ V^{-1}\ S^{-1}$)	7.31×10^{-4}	1.47×10^{-6}	0.20
Nitrite			
t_m (min)	3.68	0.024	0.67
μ_{obs} ($cm^2\ V^{-1}\ S^{-1}$)	5.16×10^{-4}	3.42×10^{-6}	0.66
μ_{eff} ($cm^2\ V^{-1}\ S^{-1}$)	1.15×10^{-4}	1.97×10^{-6}	1.71

Running electrolyte, 50 mM phosphate buffer, pH 2.5; applied voltage, −25 kV; temperature, 30 °C; UV detection at 214 nm.
[a] Migration time, mean value of nine repeated injections.

To establish the optimum conditions for the capillary electrophoretic separation of the anions of our interest, the influence of temperature, applied voltage and methanol added to the running electrolyte on anion migration and separation efficiency was investigated applying a chemometric study based on the use of the experimental design.

A 2^3 two-levels full factorial design (FFD) was initially performed [22], followed by an *F*-test for the valuation of relevant quadratic effects and measurements corresponding to a star design [23]. On the basis of the obtained regression models, the optimal separation conditions were then calculated by the multicriteria decision method of the desirability functions [24]. These conditions were found in correspondence with a temperature of 30 °C, a voltage of −20 kV and 24% (v/v) of methanol added to the running electrolyte. The addition of an organic solvent such as methanol to the running electrolyte was effective for decreasing EOF and to improve selectivity.

3.4. Method validation

Using the optimized method, a mixture of formic, malonic, succinic, acetic, lactic, 2-hydroxybutyric propionic and butyric acid were resolved each other and from sulfate, which was present in all the analyzed matrices. The analytes were identified by comparison with the reference samples and by measurement of their migration time and effective electrophoretic mobility. A mixture containing the above mentioned anions was analyzed six times sequentially. This operation was repeated over 5 days. Data regarding the R.S.D.s for the migration time and effective electrophoretic mobility of the anions of interest are summarized in Table 2.

Detection response measured for all SCAs was linearly correlated with the sample concentration injected over a range of 3–250 μg mL^{-1} (from 5 to 450 μg mL^{-1} for acetic acid). Higher concentration were not assayed because we

Table 2
Repeatability of migration time (t_m), effective mobility (μ_{eff})

Analyte (acid)	t_m[a] (min)	S.D. (min)	R.S.D (%)	μ_{eff}[b]	S.D.	R.S.D. (%)
Formic	6.69	0.126	1.87	2.56	0.010	0.55
Malonic	7.39	0.148	2.00	2.21	0.011	0.50
Succinic	7.95	0.175	2.20	1.98	0.012	0.61
Acetic	8.61	0.214	2.48	1.73	0.011	0.63
Lactic	8.77	0.219	2.50	1.68	0.006	0.36
2-Hydroxybutyric	9.29	0.252	2.71	2.69	0.020	0.74
Propionic	9.76	0.272	2.78	1.39	0.014	0.79
Butyric	10.15	0.279	2.75	1.29	0.005	0.39

[a] Migration time, mean value of six repeated injections.
[b] Effective electrophoretic mobility ($cm^2\ V^{-1}\ S^{-1}$) $\times\ 10^{-4}$.

considered that the range was wide enough for the proposed applications. The linearity was estimated from repeated injections at five different concentrations of each analyte. The linearity of the present method for all analytes was good, with correlation coefficients higher than 0.995. The linearity was achieved without an internal standard, which would have increased the correlation coefficient, as demonstrated in the application of SCFAs quantitation.

The limit of detection (LOD) was considered as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and it was ranging from 0.25 $\mu g\,mL^{-1}$ of acetic acid to 0.87 $\mu g\,mL^{-1}$ of lactic acid. The limit of quantifications (LOQ), determined at a signal/noise ratio of 10, were from 0.83 to 2.88 $\mu g\,mL^{-1}$.

The internal standard method was used for quantitative analysis. Malonic acid was selected as the internal standard because it is not present in the analyzed samples, it is completely resolved in the electropherogram from the other anions, and it migrated close to the peaks of interest. The method linearity was assessed by preparing five calibration standards in concentrations ranging from 3 to 80 $\mu g\,mL^{-1}$ for formic, succinic, propionic 2-hydroxybutyric and butyric acid, from 5 to 200 $\mu g\,mL^{-1}$ for lactic acid and from 10 to 450 $\mu g\,mL^{-1}$ for acetic acid. Calibration curves were built by plotting the relative peak areas (analyte-to-IS ratio) as a function of the standard concentration, showing excellent linearity over the considered concentration ranges, with correlation coefficients better than 0.997 and nearly passed through the origin. The precision of the method was studied as intra- and inter-day assay at three concentration levels for each analyzed compound. Day-to-day variation was assessed by analyzing replicates of standard with the same concentration on three separate days. The method was found to be precise with R.S.D. values within 1.78–3.28%, for intraday assay. Inter-day R.S.D.s were below 3.5% for all analytes. The accuracy of the method was established by determining the recovery of SCFAs spiked to the sample in the range of 20–60% of the original concentration found in faecal cultures and analyzing

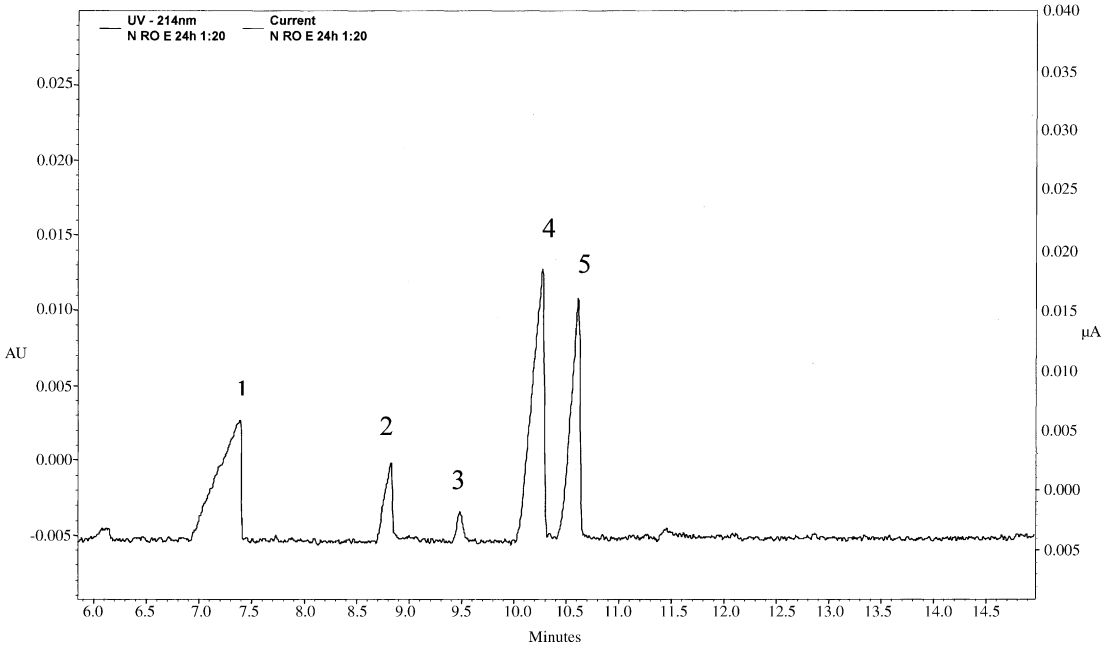

Fig. 6. Electropherogram of SCFAs produced on a faecal culture growth on Raftilose P95. Electrophoretic conditions as in the text. Peak identification: (1) sulphate (from the minimal synthetic medium), (2) malonic acid (I:S), (3) succinic acid, (4) acetic acid, (5) lactic acid.

Table 3
SCFAs produced in a faecal culture growth on Raftilose P95

Analite	Time 0[a]	Time 9 h[a]	Time 24 h[a]
Formic	n.d.	n.d.	n.d.
Succinic	80.64 (±2.28)	248.98 (±2.96)	306.66 (±8.04)
Acetic	728.74 (±9.78)	1205.45 (±3.38)	2941.73 (±28.38)
Lactic	336.42 (±4.11)	886.34 (±3.76)	2902.47 (±7.18)
2-Hydroxybutyric	n.d.	96.17 (±1.67)	n.d.
Propionic	139.30 (±3.27)	147.15 (±4.21)	77.74 (±1.03)
Butyric	161.48 (±3.03)	151.06 (±2.51)	n.d.

[a] Expressed as $\mu g\,mL^{-1}$ present in the faecal slurries.

them in triplicate according to the proposed method. In all analyzed samples mean recovery for each concentration ranged from 95.18 to 103.85% and the relative standard deviation of the results for each concentration was less than 3.50%.

3.5. Quantification of SCFA produced by faecal cultures growing on FOS or inulin

SCFA produced in faecal cultures growing on FOS (Raftilose P95) or inulin (Raftiline HP) as sole carbon sources were determined to compare the metabolic activity of intestinal microflora on differently lengthened fructans. In fact these compounds contribute to low the pH of the intestinal content, repressing pathogen bacteria and their detrimental metabolic activities. Furthermore, propionic and butyric acids are of paramount importance as colonocytes carbon sources, exerting a key role in the prevention of colonic inflammatory deaseases.

A typical electropherogram regarding SCFAs produced on a faecal culture growth on Raftilose P95 and obtained under the optimized conditions is depicted in Fig. 6. The values of SCFAs produced in a faecal culture growth on Raftilose P95 and on Raftiline HP are summarized in Tables 3 and 4, respectively. In Fig. 7 are depicted the trend of acetic and lactic acid produced on FOS and inulin, respectively. It is evident that their production is higher on FOS, according to the major increase in bifidobacteria concentration on FOS respect to inulin. As mentioned, bifidobacteria produce lactic and acetic acids as primary metabolites during carbohydrate fermentation. Therefore, lactic and acetic acids can be successfully used as markers for growth of probiotic bacteria in faecal cultures.

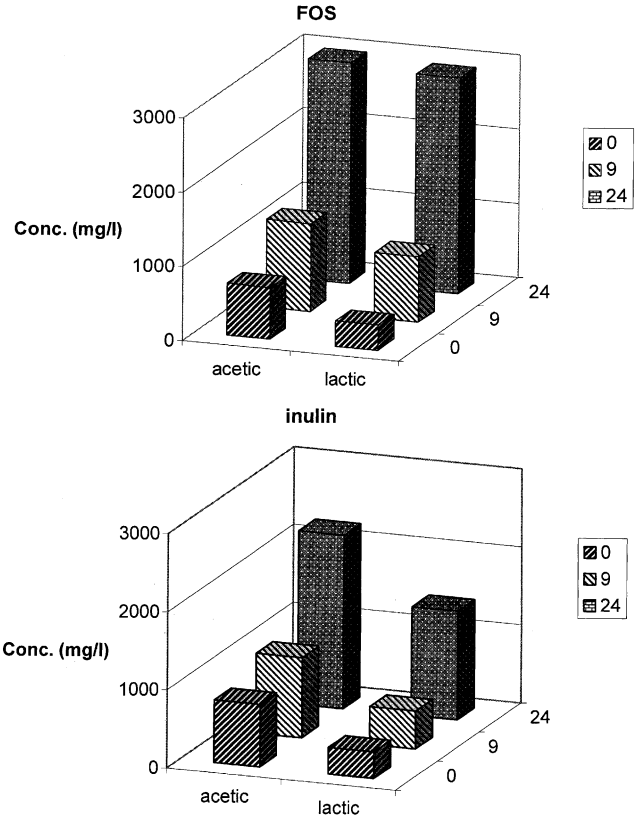

Fig. 7. Histograms presenting the kinetic and production patterns of acetic and lactic acid produced on FOS (raftilose P95) and inulin (Raftiline HP). Histograms are referred at time 0, 9, and 24 h.

Table 4
SCFAs produced in a faecal culture growth on Raftiline HP

Analite	Time 0[a]	Time 9 h[a]	Time 24 h[a]
Formic	–		110.15 (±2.37)
Succinic	94.73 (±2.75)	407.06 (±11.38)	–
Acetic	835.10 (±8.58)	1042.77 (±25.65)	2212.27 (±11.89)
Lactic	342.65 (±2.65)	490.07 (±4.18)	1390.46 (±18.92)
2-Hydroxybutyric	–	170.52 (±5.27)	916.08 (±12.66)
Propionic	–	130.51 (±3.84)	131.07 (±4.05)
Butyric	–	138.77 (±3.15)	138.06 (±1.84)

[a] Expressed as $\mu g\,mL^{-1}$ present in the faecal slurry.

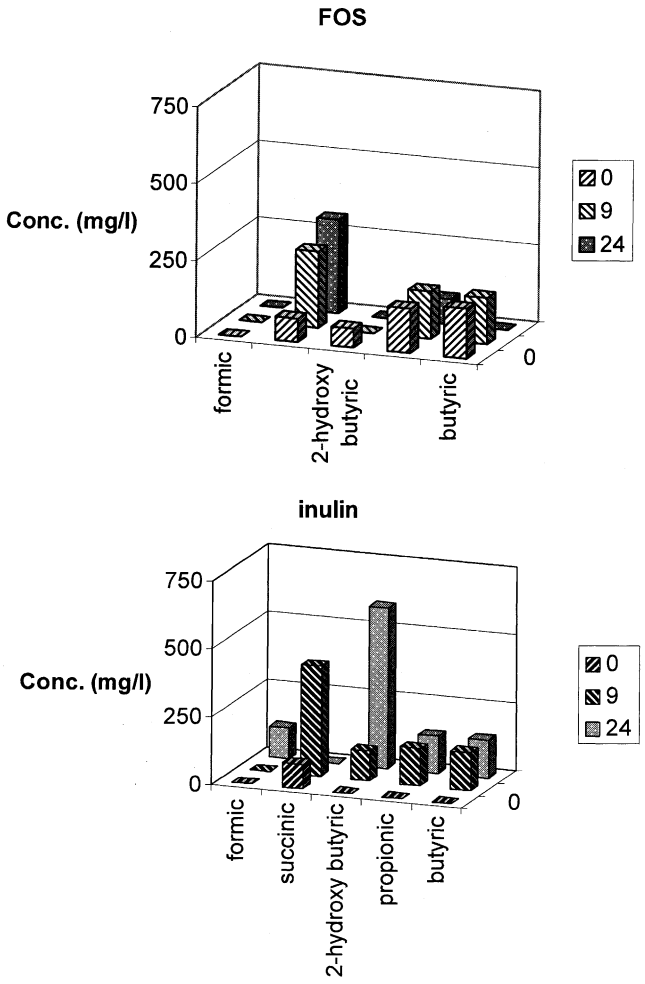

Fig. 8. Histograms presenting the kinetic and production patterns of formic, succinic, 2-hydroxybutyric, propionic and butyric acid produced on FOS (raftilose P95) and inulin (Raftiline HP). As in Fig. 7, histograms are referred at time 0, 9, and 24 h.

Formic, succinic, propionic, butyric and 2-hydroxybutyric acids were produced in lower amounts respect to lactic and acetic acids. Comparison of the trends (Fig. 8) respect to the carbon source suggests different kinetic and production patterns: inulin leads to higher final yields for formic, propionic, butyric and especially 2-hydroxybutyric acids, whereas higher amounts of succinic acid are obtained on FOS. These data suggest that differently lengthened fructans, such as FOS and long chain inulin, potentially perform differentiated effects on the colonic environment, then on human health.

4. Conclusion

HPAEC coupled with PAD detection has been demonstrated to be a powerful tool to evaluate changing in chain length distribution of FOS and inulin fermented by bifidobacteria, providing significant information about their prebiotic capabilities. Moreover, SCFAs produced in faecal cultures on FOS and inulin were quantified by CZE inco-electroosmotic conditions using hexadimethrine as dynamic coating agent and employing a benzoate-based background electrolyte and indirect UV detection at 214 nm. To improve selectivity and resolution and for controlling the electroosmotic flow, electrophoretic conditions were optimized applying a chemometric study based on the use of the experimental design.

In conclusion, the results obtained showed that both HPAEC-PAD and CZE methods could be conveniently applied in the characterization of FOS and inulins at different degree of polymerization to evaluate their prebiotic properties.

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