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In vitro fermentation of agroindustrial by-products: grapefruit albedo and peel, cactus pear peel and pineapple peel by lactic acid bacteria

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Abstract

Agroindustrial by-products from fruit processing represent an environmental issue since as organic matter their disposal could lead to their fermentation. Although some of these are employed as cattle feed or for compost, most of these by-products are not employed. These materials are an important source of bioactive compounds that can be used as fiber or carbon source in the growth of probiotic microorganisms. The objective of this work was to evaluate the prebiotic activity of four flours obtained from agroindustrial by-products (grapefruit albedo and peel, cactus pear peel and pineapple peel) with two lactic acid bacteria strains (*P. pentosaceus* UAM21 and *A. viridans* UAM22), strains with probiotic potential. Growth kinetics showed a good viability of the employed strains during the fermentation period employing the alternative carbon sources. At higher grapefruit peel flour concentration the specific growth rate was higher, and with a lower duplication time. Short chain fatty acids production confirms the prebiotic potential of these flours, since they can be employed as functional ingredient in foods.

Introduction

Agroindustrial sub-products are solid organic residues produced during fruits harvest, commercialization, process and preparation for human consumption. Agroindustrial sub-products are composed of non-edible parts of the fruits, like peel and seeds, with a varied chemical composition rich in non-digestible carbohydrates (oligosaccharides and fiber) and antioxidant compounds (Kuan and Liong, 2008). By-products generated by the fruit juice processing industry are a good source of dietary fiber and prebiotics as novel food ingredients (Lario *et al*., 2004). Functional ingredients as fiber, oligosaccharides (prebiotics) and antioxidants are more abundant in citrus peel than in juice (Moraes-Crizel *et al.*, 2013). On other hand, Opuntia ficus or cactus pear peel is an important source of carbohydrates and antioxidant compounds (Ramadán and Mörcel, 2003; Cerezal and Duarte, 2005). In same manner, pineapple peel is rich in cellulose, hemicellulose and other carbohydrates that can be source of dietary fiber (Tran and Mitchel, 1995; Rani and Nand, 2004; Aida *et al.*, 2011). Main compounds employed as prebiotic in foods are mainly non-digestible oligosaccharides that can be fermented by lactic acid bacteria (Rastall and Maitin, 2002). These compounds are linked to gastrointestinal microflora modification increasing beneficial microorganisms (probiotics mainly) and inhibiting pathogen proliferation (Swennen *et al*., 2006). Nonetheless, information about the in vitro fermentation of these novel ingredients is not available. To know the prebiotic potential of these resources is important to evaluate their prebiotic activity in order to extrapolate this information to in vivo studies in order to ensure their prebiotic activity and their inclusion in processed food products.

The objective of this work was to evaluate the prebiotic activity of four agroindustrial sub-products as grapefruit *(Citrus paradisi)* peel and albedo flour, *Opuntia ficus* or cactus pear peel flour and pineapple *(Ananas comosus)* peel flour employing lactic acid bacteria.

Materials and Methods

Sub-products process

Agroindustrial by-producs, i.e., peels and albedo from fruit processed to obtain fresh juice, were employed to obtain an added-value functional ingredient. From grapefruit *(Citrus paradise)* albedo and peel were employed. *Opuntia ficus* fruit peel and pineapple peel were also employed. Peels were collected weekly after fruit peeling and transported to University campus in plastic boxes (approximately 2 kg of each one), washed in cold tap and stored under

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refrigeration (5±1°C) until processing. Grapefruit albedo was obtained separating the white sponge tissue –albedo– manually from the peel and scalded in a NaCl (15% w/v) solution at 90°C during 30 min to eliminate limoline, removing the excess of water manually pressing. Fruit peels and albedo were cut in small 2x2 cm pieces and dried at 60°C during approximately 24 h in an air convection oven (Craft Instrumentos Científicos, México City). Dried peels and albedo were grounded in mill and sieved consecutively in No. 100, 80, 50 and 20 sieves to obtain a regular and homogeneous powder named flour. Flours were stored in hermetically dark containers until their use.

Prebiotic activity evaluation

Two lactic acid bacteria previously reported as thermotolerant with probiotic potential (Ramírez-Chavarín *et al*., 2010; Ramírez-Chavarín *et al*., 2013), *Pediococcus pentosaceus* UAM22 and *Aerococcus viridans* UAM21, were employed together with the probiotic strain *Lactobacillus rhamnosus* GG. Lactic acid bacteria strains were reactivated in MRS broth at 37°C during 24 h until obtain an optical density close to one (λ=600 nm), corresponding to approximately 10^7 CFU/mL.

Fermentations were performed adapting the methodology reported by Bustamante *et al.* (2006). Culture mean were formulated employing the different flours as carbon source to evaluate their effect on growth and acidification of the different strains. Culture medium was composed by 0.5% casein peptone (w/v), 0.3% yeast extract and carbon source at three different concentrations (0.5, 1.0 and 1.5%, w/v). Glucose was employed as control and the amount of the different flours (grapefruit albedo flour, grapefruit peel flour, cactus pear peel flour and pineapple peel flour), as alternative carbon source, was calculated according to the total soluble sugars content (Dubois *et al*., 1956) in each flour. Strains (10 mL with 10^7 CFU/mL) were inoculated in 90 mL of the different culture mediums serological flask (100 mL) and incubated at 37°C. Fermentations were monitored during 10 h, sampling each hour to determine viable count of each strains and the pH with a Beckman 50 pH meter (Beckman Coulter, Palo Alto, California).

Bacterial growth parameters were determined by standard plate count in their respective culture medium, making the pertinent dilutions, incubating at 37°C during 24 h, calculating mean growth rate constant *k* and mean duplication time g were determined according to Willey *et al.* (2008) equations,:

$$k = \frac{\mathrm{Log}N_t - \mathrm{Log}N_0}{\mathrm{Log}\ 2(t)}$$

Where:

t = time (h)

N_t = CFU/mL at the end of the exponential phase (final number)

N_0 = CFU/mL at the start of the exponential phase (initial number)

And for duplication time:

$$g = \frac{1}{k}$$

Carbohydrate consumption

Total carbohydrates content was quantified during the different fermentation employing the methodology proposed by Dubois *et al.* (1956). Samples were taken at 0, 4 and 7 h and centrifuged (2,000 x g, 15 min) to remove biomass. Supernatant was diluted to reach the range of the standard curve (0-100 mg/mL). One mL of sample was treated with 5 mL of concentrated H_2SO_4 and 1 mL of phenol solution (5%, w/v). After 15 minutes F, samples were cooled and the absorbance was taken at 519 nm, expressing sugar concentration in g/L. Consumed carbohydrates was calculated considering the initial concentration at the beginning of the fermentations.

Lactic acid and short chain fatty acids production

Lactic acid and short chain fatty acids as main metabolic primary products were determined in the fermentations with 1% of fermentable sugars, following the recommendations of Desai *et al.* (2004). Samples were taken at 0, 4 and 7 h during the fermentations. Lactic acid was determined in a Perkin Elmer 250 HPLC equipped with a Rezex ROA column (300 x 7.8 mm) (Phenomenex, Rezek), using water as mobile phase and 0.6 mL/min at 50°C and a 480 psi pressure, equipped with a light scattering detector (PL-ELS-1000, Polymer Laboratories) at 110°C. Retention times and concentration areas was calculated with a lactic acid standard. Acetic, propionic, isobutyric and butyric acids were determined by gas chromatography in a HP5890 GC equipped with a flame ionizer with and Superox FA AT-1000 column (10m x 0.25 mm). The ramp temperature was from 90 to 120°C @ 5 °C/min, employing N_2 as carrier gas at 1 mL/min, injecting 50 µL, with an injection temperature of 130 °C and a detector temperature of 150°C. Retention times and concentration areas was calculated with a standard mix of the mentioned fatty acids in a range of 0-1000 ppm.

Experimental design

The effect of the different variables (type and concentration of carbon source) on the response variables (growth and metabolite production) was evaluated according to the model:

$$y_{ij}=\mu+\alpha_i+\beta_j+\epsilon_{ij}$$

Where y_{ij} represents the response variable for the *i*-th type of carbon source (grapefruit albedo flour, grapefruit peel flour, *Opuntia ficus* fruit peel flour and pineapple peel flour) at the *j*-th concentration (0, 0.5 and 1%); μ is the overall mean; α_i and β_j are the main effects of type and concentration of carbon source, respectively; and ϵ_{ij} represents the residual error or error terms assumed to be normally distributed with zero mean and variance σ^2 (Der and Everitt, 2002). The results were analyzed by a one-way ANOVA and the significantly differences between the treatments were determined by Tukey-Kramer mean tests in the NCSS 2000 software.

Results and discussion

Cellular growth and pH

The growth kinetics for *P. pentosaceus* with 1.5% of grapefruit albedo as carbon source showed a higher growth as compared to glucose. Nonetheless, when other albedo grapefruit concentrations were employed (0.5% or 1.0%) the cellular growth was similar to control (glucose as carbon source) with final values of 7.98, 7.96 and 8.6 Log CFU/mL for 0.5, 1.0 and 1.5% of albedo grapefruit flour concentration, respectively (Fig. 1a). In *A. viridans* kinetics no difference in the viable count was observed in regard of albedo grapefruit flour concentration. This lactic acid bacteria growth was similar to control fermentation, reaching 7.94, 8.13 and 8.08 Log CFC/mL counts for 0.5%, 1.0% and 1.5% of albedo grapefruit flour concentration (Fig. 1b).

When grapefruit peel flour was employed as carbon source, the growth profile of *P. pentosaceus* and *A. viridans* was similar between each other, with a notable increase in biomass production as compared to glucose fermentations. The higher growth was observed when 1.0% of grapefruit peel flour was employed with 9.02 CFU/mL (Fig. 1c). For *A. viridans* the higher growth was observed with 1.5% of grapefruit peel flour (8.9 CFU/mL) (Fig 1d). Sendra *et al.* (2008) reported a similar behavior of other lactic acid bacteria employing lemon and orange fiber as carbon source, demonstrating that citrus by-products can be employed as carbon source for *in vitro* fermentations.

When cactus pear peel flour was employed, of *P. pentosaceus* and *A. viridans* presented higher growth at 0.5 and 1.0% of this carbon source, as compared with glucose or higher flour concentration. After the fermentation the *P. pentosaceus* and *A. viridans* count was 9.0 and 8.6 Log CFU/mL, respectively (Fig. 1e and 1f).

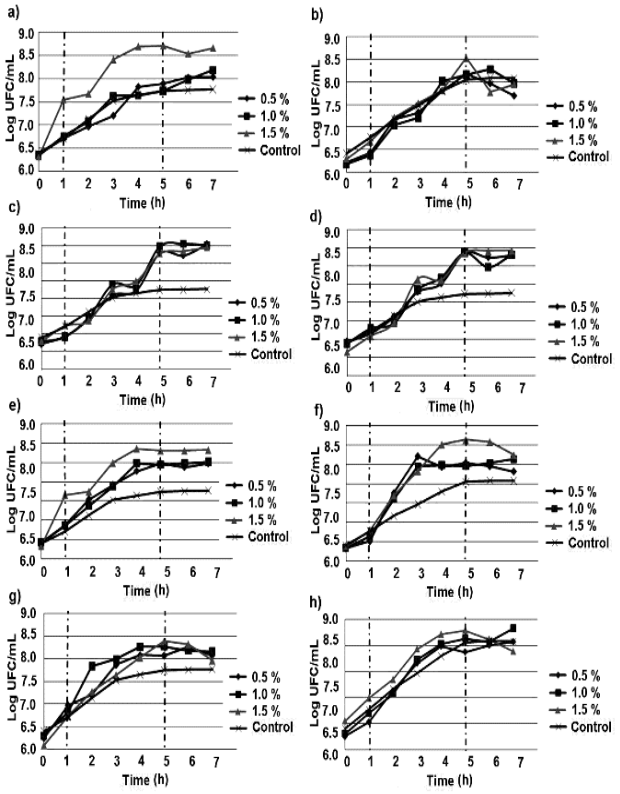

Figure 1. Cellular growth at different concentration of grapefruit albedo flour for (a) *P. pentosaceus* and (b) *A. viridans*; Grapefruit peel flour for (c) *P. pentosaceus* and (d) *A. viridans*; *Opuntia ficus* fruit peel flour for (e) *P. pentosaceus* and (f) *A. viridans*; and pineapple peel flour for (g) *P. pentosaceus* and (h) *A. viridans*.

In the fermentations with pineapple peel flour *P. pentosaceus* presented a higher growth with no appreciable difference due to carbon source concentration but higher than fermentations with glucose as carbon source (Fig. 1g). For *A. viridans* the kinetics reach higher cellular growth with 1.0% of pineapple peel flour (8.2 Log CFU/mL) as compared to glucose as carbon source (Fig. 1h).

In all the fermentation an inflexion point at the 4th hour of fermentation was observed resulting in a sigmoid curve possibly associated to the depletion of substrate and cellular the adaptation to a second carbohydrate (diauxic growth). Diauxic growth occurs when the microbial cells are cultured in batch fermentations with a mix of two carbon sources, and this type of cellular growth is characterized for two exponential phases separated by a lag phase, called diauxic lag phase (Monod, 1974). This adaptation time is the time that the microorganism needs to activate the

Table 1. Kinetic parameters for *P. pentosaceus* with the different carbon sources.

Concentration	Specific growth rate, k (h^{-1})				Duplication time, g (h)			
	Grapefruit albedo	Grapefruit peel	*Opuntia ficus* fruit peel	Pineapple peel	Grapefruit albedo	Grapefruit peel	*Opuntia ficus* fruit peel	Pineapple peel
Control	0.89±0.04 $^{c, C}$	0.89±0.04 $^{a, C}$	0.89±0.04 $^{b, C}$	0.89±0.04 $^{b, C}$	1.12±0.05 $^{d, D}$	1.12±0.05 $^{f, D}$	1.12±0.05 $^{g, D}$	1.12±0.05 $^{g, D}$
0.5 %	1.02±0.09 $^{c, B}$	1.73±0.07 $^{a, B}$	1.36±0.07 $^{b, B}$	1.21±0.04 $^{b, B}$	0.99±0.09 $^{d, F}$	0.58±0.02 $^{f, F}$	0.74±0.04 $^{g, F}$	0.83±0.03 $^{g, F}$
1.0 %	0.92±0.16 $^{c, B}$	1.80±0.05 $^{a, B}$	1.34±0.08 $^{b, B}$	1.31±0.02 $^{b, B}$	1.11±0.19 $^{d, E}$	0.55±0.01 $^{f, E}$	0.75±0.05 $^{g, E}$	0.76±0.01 $^{g, E}$
1.5 %	1.59±0.03 $^{c, A}$	1.66±0.07 $^{a, A}$	1.64±0.02 $^{b, A}$	1.53±0.11 $^{b, A}$	0.63±0.01 $^{d, G}$	0.60±0.03 $^{f, G}$	0.61±0.01 $^{g, G}$	0.66±0.04 $^{g, G}$

$^{a, b, c}$ means with same letter in same row are not significantly ($p<0.05$) different for k at the different carbon source.
$^{A, B, C}$ means with same letter in same column are not significantly ($p<0.05$) different for k at different concentration.
$^{d, e, f}$ means with same letter in same row are not significantly ($p<0.05$) different for g for the different carbon source.
$^{D, E, F, G}$ means with same letter in same column are not significantly ($p<0.05$) different for g for the different concentration.

Table 2. Kinetic parameters for *A. viridans* with the different carbon sources

Concentration	*k* (h^{-1})				g (h)			
	Grapefruit albedo	Grapefruit peel	*Opuntia ficus* fruit peel	Pineapple peel	Grapefruit albedo	Grapefruit peel	*Opuntia ficus* fruit peel	Pineapple peel
Control	1.09±0.21 $^{c, C}$	1.09±0.21 $^{a, C}$	1.09±0.21 $^{b, C}$	1.09±0.21 $^{d, C}$	0.95±0.18 $^{f, D}$	0.95±0.18 $^{g, D}$	0.95±0.18 $^{g, D}$	0.95±0.18 $^{g, D}$
0.5 %	1.30±0.10 $^{c, B}$	1.62±0.07 $^{a, B}$	1.47±0.07 $^{b, B}$	1.07±0.08 $^{d, B}$	0.77±0.06 $^{f, E}$	0.62±0.02 $^{g, E}$	0.68±0.03 $^{g, E}$	0.94±0.06 $^{g, E}$
1.0 %	1.32±0.04 $^{c, B}$	1.65±0.04 $^{a, B}$	1.41±0.21 $^{b, B}$	1.21±0.17 $^{d, B}$	0.76±0.02 $^{f, E}$	0.61±0.02 $^{g, E}$	0.72±0.11 $^{g, E}$	0.84±0.12 $^{E, E}$
1.5 %	1.48±0.08 $^{c, A}$	1.80±0.10 $^{a, A}$	1.83±0.08 $^{b, A}$	1.15±0.28 $^{d, A}$	0.68±0.04 $^{f F}$	0.56±0.03 $^{g, F}$	0.55±0.02 $^{g, F}$	0.91±0.22 $^{E, F}$

a, b, c, d means with same letter in same row are not significantly ($p<0.05$) different for k at the different carbon source.
A, B, C means with same letter in same column are not significantly ($p<0.05$) different for k at different concentration.
e, f, g means with same letter in same row are not significantly ($p<0.05$) different for g for the different carbon source.
D, E, F means with same letter in same column are not significantly ($p<0.05$) different for g for the different concentration.

necessary enzymes to the consumption of the second carbohydrate, and the consumption preference for the substrates depends on the microorganism affinity for the substrate and the enzymatic availability to metabolize the substrate (Jacob and Monod, 1961). In general, the growth kinetics employing the different flours as carbon source presented viable count values between 8.0-9.0 CFU/mL, higher values than the obtained with glucose as carbon source. These data suggest that the oligosaccharides present in the flours can be fermented by the lactic acid bacteria.

During fermentations with the different carbon sources the pH range during the lag phase was from 6.5 to 4.4, depending on the carbon source and concentration. In general, the acidification profile followed the same pattern, i.e., adaptation phase (0 to 1 h), exponential phase (1 to 5 h) and lag phase (5 to 7 h). Glucose fermentation started with a pH= 6.5, reaching a final pH= 4.8. Grapefruit albedo as carbon source the final pH was higher than for control, 6.3±0.1, 6.1±0.1 and 6.4±0.2 with 0.5%, 1.0% and 1.5%, respectively, for *P. pentosaceus* (Fig. 2a); and 6.05±0.2, 6.0±0.2 and 5.8±0.2 with 0.5%, 1.0% and 1.5%, respectively, for *A. viridans* (Fig. 2b). This higher pH could be due probably to a lower acidification (as lactic acid or other short chain organic acids). When grapefruit flour was employed as carbon source, *P. pentosaceus* and *A. viridans* presented a similar acidification profile, with pH as lower as the obtained with glucose when flour concentration was 1.0 or 1.5%. Final pH values of 5.7±0.3, 5.1±0.4 and 4.8±0.3 were obtained with 0.5%, 1.0% and 1.5%, respectively, for *P. pentosaceus* (Fig. 2c); and 5.4±0.2, 4.9±0.3 and 4.7±0.2 were

Table 3. Organic acids production for the different carbon sources (1.0%, w/v)

	Lactic (g/L)		Acetic (g/L)		Butyric (g/L)	
	P. pentosaceus	*A. viridans*	*P. pentosaceus*	*A. viridans*	*P. pentosaceus*	*A. viridans*
Control	3.50±0.30 $^{a, A}$	2.8±0.01 $^{a, B}$	0.138±0.0015 $^{e, A}$	0.070±0.001 $^{d, B}$	0.054±0.001 $^{a, A}$	0.034±0.9 $^{a, B}$
Grapefruit albedo	2.04±0.05 $^{b, A}$	2.2±0.01 $^{c, B}$	0.204±0.0026 $^{b, A}$	0.186±0.002 $^{c, B}$	0.008±0.002 $^{b, A}$	0.0000 $^{c, B}$
Grapefruit peel	2.38±0.16 $^{b, A}$	2.1±0.01 $^{d, B}$	0.191±0.0025 $^{c, A}$	0.406±0.005 $^{b, B}$	0.006±0.001 $^{c, A}$	0.009±0.001 $^{b, B}$
Pineapple peel	2.25±0.07 $^{b, A}$	2.4±0.02 $^{b, B}$	0.154±0.0053 $^{d, A}$	1.156±0.012 $^{a, B}$	0.0000 $^{d, A}$	0.0000 $^{c, B}$
Opuntia ficus fruit peel	2.08±0.14 $^{b, A}$	2.1±0.03 $^{d, B}$	0.274±0.0020 $^{a, A}$	0.199±0.007 $^{c, B}$	0.0000 $^{d, A}$	0.0000 $^{c, B}$

$^{a, b, c, d, e}$ means with same letter in same column are not significantly ($p<0.05$) different for the carbon source
$^{A, B}$ means with same letter in same row are not significantly ($p<0.05$) different strain

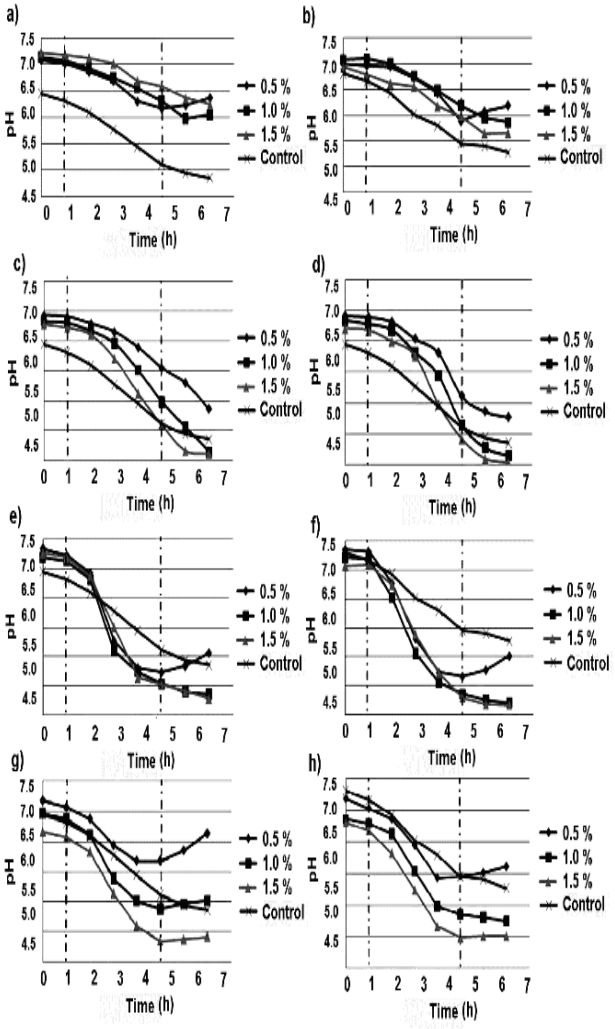

Figure 2. Fermentation pH at different concentration of grapefruit albedo flour for (a) *P. pentosaceus* and (b) *A. viridans*; Grapefruit peel flour for (c) *P. pentosaceus* and (d) *A. viridans*; *Opuntia ficus* fruit peel flour for (e) *P. pentosaceus* and (f) *A. viridans*; and pineapple peel flour for (g) *P. pentosaceus* and (h) *A. viridans.*

obtained with 0.5%, 1.0% and 1.5%, respectively, for *A. viridans* (Fig. 2d). For *Opuntia ficus* fruit peel flour fermentations an acidification close to control (glucose) was observed for both microorganisms when carbon source concentration was 1.0%. Final pH values of 4.9±0.2, 4.4±0.1 and 4.4±0.2 were obtained with 0.5%, 1.0% and 1.5%, respectively, for *P. pentosaceus* (Fig. 2e); and 4.8±0.2, 4.3±0.1 and 4.2±0.1 were obtained with 0.5%, 1.0% and 1.5%, respectively, for *A. viridans* (Fig. 2e). Pineapple peel flour fermentations resulted in higher pH values as compared with the other carbon sources, where glucose resulted in the lower pH values. Final pH values of 5.9±0.2, 4.9±0.1 and 4.4±0.2 were obtained with 0.5%, 1.0% and 1.5%, respectively, for *P. pentosaceus* (Fig. 2f); and 5.5±0.1, 4.8±0.1 and 4.5±0.1 were obtained with 0.5%, 1.0% and 1.5%, respectively, for *A. viridans* (Fig. 2g).

Growth kinetic parameters

Table 1 show the specific growth rate and duplication time for the different fermentations and *P. Pentosaceus*. When grapefruit peel flour was employed as carbon source, the specific growth rate was significantly ($p<0.05$) higher than for the other flours or glucose as carbon source. High flour concentration (1.5%) resulted as well in significantly higher specific growth rate. For the duplication time, the significantly ($p<0.05$) lower values was observed when grapefruit peel flour was employed as carbon source. In same manner, higher flour concentrations (1.5%) resulted as well in significantly ($p<0.05$) lower g values.

Fermentations with *A. viridans* are show in Table 2. Specific growth rate was significantly ($P<0.05$) higher with grapefruit peel flour. As in the case of *P. pentosaceus* fermentations, to employ higher flours concentrations (1.5%) resulted in significantly ($p<0.05$) higher k values. Duplication time was significantly ($p<0.05$) lower for *Opuntia ficus* fruit peel flour and grapefruit peel flour. Nonetheless, duplication time was significantly lower ($p<0.05$)

in glucose fermentations, followed by 1.5% flour concentration.

Organic acids production

Organic acid production was affected by the type of carbon source. In *P. pentosaceus* fermentations, lactic acid production was significantly ($p<0.05$) higher in glucose samples than the fermentation employing another carbon source. Acetic acid was significantly ($p<0.05$) higher in *Opuntia ficus* fruit peel flour, and the lower amount detected was in control samples. Butyric acid was significantly ($p<0.05$) higher in control samples, followed by grapefruit albedo flour and grapefruit peel flour. In Pineapple flour and *Opuntia ficus* fruit peel flour fermentations no detection of this organic acid was found (Table 3).

In *A. viridans* fermentations, carbon source affected as well the organic acids production. Control samples employing glucose as carbon source resulted in significantly ($p<0.05$) higher lactic acid values, followed by pineapple flour. Acetic acid production was significantly ($p<0.05$) higher when pineapple was employed as carbon source, and the lower values was detected in control samples. Propionic acid was only detected in grapefruit albedo flour fermentations. Butyric acid was only detected in control and grapefruit peel flour samples (Table 3).

P. pentosaceus produced significantly ($p<0.05$) higher amount of lactic, acetic and butyric acids. Lactic acid was the major acid produced (>95%), corroborating the homofermentative fermentation of these strains.

These results and the growth profiles suggest that the presence of alternative compounds that can be employed as carbon source for the lactic acid bacteria affected medium pH and the secondary metabolites production as SCFA, as a result of the fiber hydrolysis contained in the different fruit peels flours. Lactic acid production had an influence on the medium pH with faster carbohydrate consumption, making difficult the propagation of other undesirable microorganism (Marklinder and Lönner, 1992). Main SCFA produced during lactic fermentations are acetate, propionate and butyrate (Nordgaard and Mortensen, 1995; Lindgren and Dobrogosz, 1990; Zwietering *et al*., 1990). The increase in the concentration of these compounds at colon level by the native microflora fermenting simple or complex carbohydrates had a beneficial impact on health, since besides the inhibition of pathogens by acidification, mineral absorption, vitamins production, glycemic index regulation and decrease of blood lipids are improved as well (Tungland and Meyer, 2002). Lactic acid bacteria are capable to metabolize other carbon sources, and the use of these carbohydrates are determinate by the chemical structure, polymerization degree and the type and composition of the monomeric units (Biedrzycka and Bielecka, 2004; Bustamante *et al.*, 2006.; Mandalari *et al*., 2007).

Conclusions

The different agroindustrial by-products evaluated in this research, as grapefruit peel flour, grapefruit albedo, cactus pear peel flour and pineapple peel flour, resulted to be a cheap and fermentable carbon source by lactic acid bacteria, with an acceptable short chain organic acids production.

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