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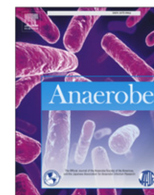

Clinical microbiology

Propionibacterium acidipropionici CRL1198 influences the production of acids and the growth of bacterial genera stimulated by inulin in a murine model of cecal slurries

M.J. Lorenzo-Pisarello [a,b], M.L. Gultemirian [a], C. Nieto-Peñalver [b], A. Perez Chaia [a,b,*]

[a] *Centro de Referencias para Lactobacilos (CERELA)-CONICET, Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina*
[b] *Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina*

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Different attempts have been made to improve the health status of humans and animals by increasing the intestinal production of short-chain fatty acids (SCFA) derived from non-digestible carbohydrates fermentation. In this paper we investigate the *in vitro* production of short-chain fatty acids (SCFA) after addition of inulin, propionibacteria or a combination of both in an experimental model of mice cecal slurries. The development of bacterial genera which are usually stimulated by inulin addition was also investigated. According to our experimental data, acetic acid and butyric acids concentrations increased after incubation in slurries that had no supplements. By contrast, butyric acid concentrations remained in the basal value when supplements were used. Fermentation of only inulin did not increase the concentration of total SCFA. *Propionibacterium acidipropionici* CRL1198 improved the production of propionic acid in cecal slurries when it was added alone, but the effect was more noticeable in the combination with inulin. A modulation of the global fermentative activity of the cecal microbiota was evidenced by the increase on the ratio propionic acid/SCFA in supplementations with propionibacteria. Statistical analysis of data demonstrated that samples from homogenates with propionibacteria alone or combined with inulin belong to the same cluster. The presence of propionibacteria limited the growth of *Bacteroides fragilis* and *Clostridium hystoliticum* groups in slurries with and without inulin. The growth of *Bifidobacterium* was not modified and the stimulating effect of inulin on lactobacilli disappeared in the presence of propionibacteria. In conclusion, dairy propionibacteria are potential candidates to develop new functional foods helpful to ensure the intestinal production of SCFA during inulin supplementation and to control the overgrowth of bacteria belonging to *Bacteroides* and *Clostridium* genera.
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1. Introduction

The large intestine of humans and monogastric animals is a complex ecosystem that harbors several hundreds of different bacterial species including autochthonous components and allochthonous members that pass through together with food and beverages [1]. In order to obtain the energy for growth and cellular maintenance, this microbiota metabolizes different exogenous carbohydrates, no digested by the host's intestinal enzymes, and endogenous substrates like mucus and epithelial or bacterial cells residues. The bacterial catabolism of carbohydrates produces organic acids like lactic acid and short-chain fatty acids (SCFA), mainly acetic, propionic and butyric acids. [2]. Concentration and ratio of SCFA are strongly influenced by the diet and the metabolism of bacterial genera that are in high number in the intestine.

Measurements of SCFA in the intestinal content only provide an indication of SCFA production as its concentration result from the equilibrium between the production by the metabolic activity of the microbiota and the absorption throughout the intestinal mucosa. The transport of SCFA from the intestinal lumen is responsible for Na and water absorption [3] in the colon. SCFA are also important energy sources for the colonocytes and have a trophic effect on the colonic mucosa [4]. Butyric acid induces cell differentiation and stimulates apoptosis of cancerous cells, as well as propionic acid [5–7]. Part of the SCFA absorbed reaches the general circulation but are rapidly cleared and metabolized in muscles and brain, providing additional energy, and in the liver where propionic acid interferes with the cholesterol synthesis. Given the beneficial effects of SCFA, different attempts have been made in order to increase the intestinal production of these acids

* Corresponding author. Centro de Referencias para Lactobacilos (CERELA)-CONICET, Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. Tel.: +54 381 4311720; fax: +54 381 4005600.
E-mail address: apchaia@cerela.org.ar (A. Perez Chaia).

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throughout fermentation of dietary carbohydrates expecting to improve the health status of consumers. For this purpose, dietary supplementation with non-digestible carbohydrates like oligofructose and fructooligosaccharides has been evaluated [8,9].

In the last years, other strategies to increase SCFA concentrations in the intestinal environment have been also considered, such as the consumption of probiotic strains which release acetic, propionic or butyric acid as final products from carbohydrates fermentation. *Saccharomyces boulardii* [10] and anaerobic bacteria like *Faecalibacterium prausnitzii* or *Butyricicoccus pullicaecorum* have been proposed for this purpose [11]. In that sense, dairy propionibacteria may be suitable to be used as probiotics for improving intestinal SCFA concentrations as they are producer of acetic and propionic acid, they are considered GRAS microorganisms (Generally Recognized As Safe) and commonly used for dairy products elaboration. It has been demonstrated *in vitro*, in mice cecal slurries, that propionibacteria may influence the overall production of SCFA, as they enhance propionic acid concentration derived from fermentation of dietary residues, lactose or carbohydrates released from mucus by the bacterial glycosidases [12]. There is also *in vivo* evidence of the survival and metabolic activity of some strains of propionibacteria in the intestine of mice and rats [12,13], gastrointestinal tract of human microbiota-associated rats [14], and humans' intestine [15,16]. However there is no information about the benefits of a simultaneous administration of dairy propionibacteria and non-digestible carbohydrates like inulin-type fructans. Our interest was to determine if cross-feeding among propionibacteria and autochthonous bacteria that metabolize fructans promotes propionic acid production during inulin supplementation and if this interferes the development and fermentative activity of the resident bacteria. The interest was focused to lactobacilli, bifidobacteria and bacteroides, which have been reported as inulin consumers and clostridia that can utilize lactic acid [17–20]. Taking into account that the degradation of fibers show good correlation between man and murine models and that these models has been previously used to predict bacterial degradation of dietary fibers in man [21], we have performed our *in vitro* assays in an experimental model of mice.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Propionibacterium acidipropionici CRL1198 (CRL: Centro de Referencia para Lactobacilos, CERELA, Tucumán, Argentina), a strain resistant to the gastrointestinal tract conditions [22,23], was selected for the present study. The strain was stored at −20 °C in 10% (w/v) reconstituted non-fat milk (NFM) supplemented with 0.5% yeast extract. Before each experiment, it was inoculated in Laptg broth [24] with the following composition: 15 g/L peptone, 10 g/L tryptone, 10 g/L yeast extract, 10 g/L glucose, 1 mL polysorbate 80, pH 6.50. It was transferred twice to fresh medium after incubations for 24 h at 37 °C.

2.2. Growth on inulin

To study the ability of *P. acidipropionici* CRL1198 to growth in media with inulin, the strain activated in Laptg broth medium was then propagated for 24 h at 37 °C in a modified Laptg broth containing 1% fructose instead of glucose. Afterwards, the cells were harvested by centrifugation (10,000 × g, 10 min, 4 °C), suspended in one tenth of the original volume of culture in fresh Lapt base medium without sugars and used to inoculate the same medium with and without 10 g/L filter-sterilized inulin (Sigma–Aldrich, Buenos Aires, Argentina). The suspension volume used to inoculate the media was 1% (v/v). The cultures were incubated at 37 °C for 12 h in an anaerobic glove box (Anaerobic System model 1024, Forma Scientific, Marietta, USA) with atmosphere of 100% N_2. The growth was followed by Absorbance at 560 nm.

2.3. Preparation of cecal slurries and in vitro fermentation experiments

A culture of *P. acidipropionici* propagated in Laptg broth medium was harvested by centrifugation (3000 × g, 10 min, 4 °C), washed with sterile saline solution (9 g/L NaCl) and suspended in one fifth of the original volume of culture in the same solution. This suspension was used to inoculate mice cecal homogenates when it was necessary.

Twelve male 6-weeks-old BALB/c mice, each weighing 25–30 g, from the inbreed colony of CERELA were housed in metal cages, three animals per each, and maintained in a room under controlled environmental conditions of 25 °C and a 12-h light-dark cycle. They were allowed free access to a conventional solid balanced diet (*Cooperación*, Asociación de Cooperativas Unidas, San Nicolás, Buenos Aires, Argentina) and water and at the day of the experiment were sacrificed by cervical dislocation. Four slurries containing a pool of the cecal content of three animals each one were obtained as previously reported [12]. They were weighed and diluted in pre-reduced sterile saline solution to adequate volume to give 5% (w/v) suspensions. Sterilized glass beads (diameter 3 mm) were added to the suspensions, which were homogenized on a vortex mixer for 2 min under anaerobic conditions. The homogenates were transferred to sterile glass bottles and then supplemented with sterile saline solution, sterile inulin to final concentration of 10 g/L of suspension (0.2 g per gram of wet weight of cecal content), bacterial suspension up to approximately 5×10^9 propionibacteria per gram of wet weight of cecal content, or a combination of propionibacteria and inulin to obtain four different fermentation mixtures. Afterwards, they were incubated without stirring for 10 h at 37 °C in the anaerobic glove box and samples were withdrawn at regular time intervals. Counts of bacteroides, clostridia, lactobacilli, bifidobacteria and *P. acidipropionici*, pH values and the concentration of fermentation products were determined.

The CERELA Committee of Ethics approved the protocol used for animal studies.

2.4. Organic acids analysis

Samples taken for organic acids analyses were acidified with H_2SO_4 0.01 mol/L, centrifuged for 30 min (3000 × *g*; 4 °C) and filtered onto 0.2 μm pore size membranes (Millipore, Massachusetts, USA). Ethanol, SCFA and lactic acid produced during fermentations were determined using a 300 × 7.8 mm Rezex ROA-organic acids column (Phenomenex, Torrance, USA) operated at 55 °C with H_2SO_4 0.01 mol/L as the mobile phase. Products concentrations were reported as mmol/g of cecal content.

2.5. Enumeration of total bacteria in cecal contents

For enumeration of bacterial populations, samples were prepared in a way similar to other reports [25–28]. Cecal homogenates were centrifuged at 700 × g for 1 min to remove large particles from the suspensions and 300 μL of sample supernatants were diluted with 900 μL of freshly prepared cold 4% paraformaldehyde solution (PFA) in 130 mmol/L NaCl, 10 mmol/L phosphate buffer pH 7.2 (PBS). Samples were fixed for 16 h at 4 °C, centrifuged (10,000 × g; 5 min; 4 °C), washed twice and suspended

in 1 mL of a mixture of ethanol 99.9%:PBS (1:1) and stored at −20 °C until required, but no longer than 3 months.

Total counts of bacteria per milliliter of suspension was obtained by staining 50 μL of a dilution of fixed cells of each sample with 5 μL of 10 μg/μL 4′,6-diamidino-2-phenylindole (DAPI) solution for 5 min in the dark. Subsequently, a standardized volume of each stained sample (15 μL) was spotted on a slide, covered with a coverslip and observed with the 100 × objective with a conventional fluorescence microscope (Leica DM LS2, Leica Microsystems, Wetzlar, Germany) fitted with an appropriated filter for DAPI. The number of fluorescent cells was determined by direct visual cell counting. For this purpose, cells from 10 microscopic fields were counted and the average number of cells per field was calculated.

The total number of cells per milliliter of suspension was determined by using a factor of 1.525×10^{-6} mL per field that represents the volume of the liquid between the slide and coverslip in the field of view under the experimental conditions used. This value was in the same order than that used by Swidsinski et al. [29] for a Nikon fluorescence microscope. The microscope factor was previously determined by counting, under the same conditions, the number of fluorescent cells per field from DAPI stained yeast suspensions with known counts in a range of 1.55×10^6 to 2.00×10^8 cell/mL. The cells number in the field of view vs number of cells per milliliter was represented in a linear graph and the slope obtained was taken as the microscope factor. The value used in subsequent experiments was the average of three independent determinations. Therefore, for direct count of bacteria per milliliter in the cecal slurries, the average number of fluorescent bacteria in a field of view was divided by the microscope factor and corrected for the dilution used. Finally, the number of bacteria per milliliter and a dilution factor were used to determine bacterial counts per gram of cecal content.

2.6. Probes for fluorescence in situ hybridization (FISH)

Oligonucleotide probes 5′end-labeled with 6-Carboxyfluorescein (6-FAM) used in the study and their target organisms are listed in Table 1. Eub338 was used to control the permeability of fixed cells and Non338 to control non-specific binding [30]. The probes used for hybridization of intestinal bacteria are the frequently reported as for this purpose in fecal samples [25–28]. The detection of *P. acidipropionici*, was performed with an oligonucleotide probe designed in this study.

Table 1
16S rRNA-targeted oligonucleotide probes.[a]

Probe	Sequence (5′– 3′)	Target organism	Reference
Eub338	GCTGCCTCCCGTAGGAGT	*Bacteria*	[56]
Non338	ACTCCTACGGGAGGCAGC	Negative control	[57]
Bfra602	GAGCCGCAAACTTTCACAA	*Bacteroides fragilis* subgroup[b]	[25]
Bdis656	CCGCCTGCCTCAAACATA	*Bacteroides distasonis* species	[25]
Chis150	TTATGCGGTATTAATCTYCCTTT	*Clostridium histolyticum* subgroup[c]	[25]
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus* and *Enterococcus*	[26]
B164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	[27]
Pap446	ACACCCCAAAACGATGCCTTCGCC	*Propionibacterium acidipropionici*	This study

[a] The probes used for hybridization of these groups of bacteria are currently used in fecal samples with the exception of Pap probe that was constructed in this study (See Materials and methods section).
[b] Positive reaction with *Bacteroides fragilis*, *vulgatus*, *eggerthii*, *thetaiotaomicron*.
[c] Specific for most species of the *histolyticum* group (*Clostridium* clusters I and II). Positive reaction with *Clostridium histolyticum*, *butyricum*, *perfringens*, *putrificum*, *cadaveris*, *botulinum*.

Genomic DNA of the strain CRL1198 was extracted from exponentially growing cultures utilizing the CTAB procedure [31]. For DNA amplification, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers were utilized according to standard protocols [32]. DNA sequencing was performed by Macrogen (Seoul, South Korea) using primers 27F and 1492R. The 16S rDNA sequence was submitted to GenBank and is registered under accession no. EU597014.

A species-specific oligonucleotide probe was designed based on the comparative analysis of 16S rDNA sequences, obtained from DDBJ/GenBank/EMBL database. The sequence of *P. acidipropionici* CRL1198 were aligned with BLASTN 2.2.19 [33] and analyzed searching for a target region which enabled discrimination with other species from the genus *Propionibacterium* and genera of anaerobic bacteria numerically important in the intestinal flora. A target site on the 16S rDNA of *P. acidipropionici* CRL1198 was selected and the corresponding probe for the rRNA was constructed. The designed oligonucleotide probe which matches with the selected target site was tested for specificity with the Probe Match function of Ribosome Database Project [34]. The probe referred to as Pap446, was assayed to determine the optimal hybridization conditions with the strain CRL1198.

Different strains of propionibacteria and other non-target genera from the CRL Collection were examined at the same conditions in a FISH protocol to confirm the probe specificity [35]. Before using Pap446 probe, it was also assayed in mice cecal slurries without propionibacteria supplementations and no signal was detected at the cecal concentration used in the present study.

All the probes used in this study were provided by Sigma–Aldrich (Buenos Aires, Argentina).

2.7. Hybridization procedure

Ten microliters of fixed cells suspension of each sample (see Section 2.5) were spotted onto assigned positions on glass slides previously coated with a gelatin suspension (0.01% $KCr(SO_4)_2$, 0.1% gelatin) [36]. They were allowed to dry at room temperature for 30 min and successively dehydrated during 3 min in 50, 80 and 96% (v/v) ethanol. Slides for each probe, including positive and negative controls (Eub338 and Non338 respectively), were prepared from the different samples.

Protocols for hybridization with probes Chis150, Lab158, Bif164, Bfra602 and Bdis656 probes were similar to other previously reported [25–28]. For hybridization with Chis150, Lab158, Bif164 and Pap446 probes, cells were permeabilized by covering the smears with 10 μL of 1 mg/mL lysozyme (Sigma–Aldrich, Argentina) in 100 mM Tris–HCl pH 7.2 for 5 min at 25 °C (Chis150, Lab158 and Bif164 probes) or 10 min at 25 °C (Pap probe). Enzymatic treatment was stopped by rinsing the slides thoroughly with sterile water. Slides were then dried and dehydrated as indicated above. For hybridization assays with Bfra602 and Bdis656 probes, lysozyme treatment was omitted.

Fifty microliters of preheated hybridization buffer (20 mM Tris–HCl; 0.9 M NaCl; 0.1% SDS; pH 7.2) containing 5 ng/μL of the appropriated probe, was dropped on the cell smears for each probe and sample. Smears were overnight incubated at the assigned temperature in a dark box humidified with hybridization buffer, with exception of smears for Pap446 probe that were incubated for 6 h. Subsequently, the slides were rinsed during 20 min in preheated washing buffer (20 mM Tris–HCl; 0.9 M NaCl; pH 7.2) and overlaid with 50 μL of a 500 ng/μL DAPI solution in sterile distilled water for 5 min in the dark. After rinsing in sterile distilled water, the slides were air-dried and stored in the dark. All the slides were covered with mounting medium (INOVA Diagnostics Inc., San

Diego, CA) and coverslips and observed with the 100 × objective and appropriated filters for DAPI and 6-FAM.

At least 10 fields from each slide, each containing 10–100 cells, were randomly selected. Fluorescent cells were counted and mean values obtained for hybridized and DAPI stained cells. The percentile value of each population in the overall flora was calculated with these data. Total bacteria per gram and the percentile of each population in the overall microbiota were used to calculate counts per gram of cecal content.

2.8. Statistical analysis

The results of Figs. 1 and 2 are expressed as the average of five independent experiments. Significant differences between means were determined by Tukey's test after analysis of variance (ANOVA) with Minitab Statistic Program, release 12 for Windows. A P value of <0.05 was considered statistically significant.

A principal component analysis (PCA) was performed on quantitative data of SCFA production obtained from cecal homogenates supplemented with inulin, propionibacteria or a mixture of both of them after 10 h of anaerobic incubation. Each independent experiment was numbered from 1 to 5 and samples of each treatment group were named as C (control), P (with propionibacteria), I (with inulin) and IP (with both supplements). Samples for PCA were identified with a character and a number to indicate the experiment and treatment to which they correspond. The variables analyzed were ethanol, formic, acetic, propionic, butyric and lactic acids. Data used were mmol/g of cecal content of each product without previous normalizing. The software package PC-ORD v.5.0 (Multivariate Analysis of Ecological Data, MjM Software Design) was used for statistical analysis and scores for variables as distance-based biplot were represented in a scatterplot.

After PCA, cluster analysis based on Euclidean distance measure, using the group linkage method of the nearest neighbor was performed. Results were plotted as two-way dendrogram using this graph tool of the PC-ORD software.

3. Results

3.1. Growth on inulin as energy source

Growth of *P. acidipropionici* CRL1198 throughout the time was studied on Lapt broth base with or without the addition of 1% inulin. The strain showed the same development during 12 h of incubation in both cultures with very low yield of biomass (data not shown). The data suggested that inulin was not used by this strain as energy source.

3.2. Microbial populations in cecal slurries

Counts of selected populations from each homogenate and time of incubation are showed in Fig. 1. The highest numbers of

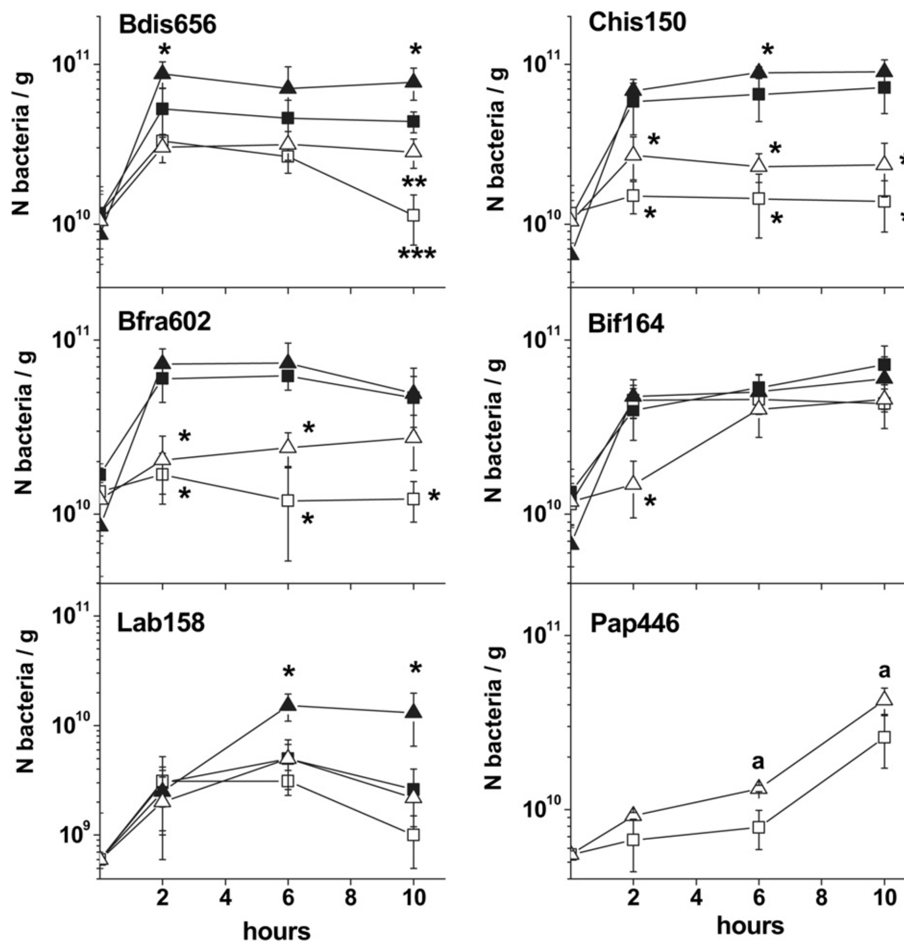

Fig. 1. Counts of selected bacterial populations in cecal homogenates supplemented or not. Bfra602: *Bacteroides fragilis* subgroup; Bdis656: *Bacteroides distasonis* species; Chis150: *Clostridium histolyticum* group; B164: genus *Bifidobacterium*; Lab158: genus *Lactobacillus*; Pap446: *Propionibacterium acidipropionici*. Symbols: (■), without additions; (▲) inulin; (□) propionibacteria; (△) combination of inulin and propionibacteria. An asterisk indicates significant differences related to the homogenate without additions at each time. The character a in propionibacteria counts indicates significant differences in the homogenate with inulin related to the homogenate without prebiotic.

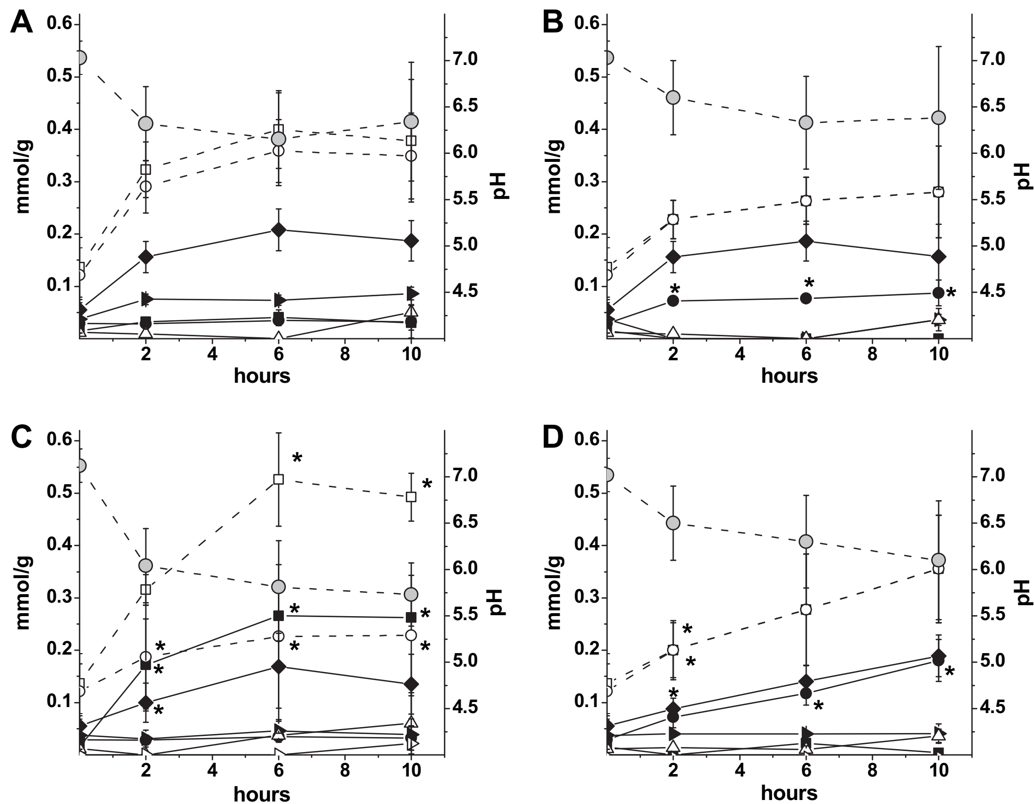

Fig. 2. Concentrations of the main organic compounds produced and pH values in cecal homogenates supplemented or not. Panel A: Without additions; Panel B: Inoculated with *P. acidipropionici* CRL1198; Panel C: With inulin; Panel D: With a combination of inulin and propionibacteria. Symbols: △, ethanol; □, total acids; ■ lactic acid; ○, SCFA; ▷, formic acid; ◆, acetic acid; ●, propionic and ►, butyric acid; ◯, pH. An asterisk indicates significant differences related to the homogenate without additions at each time.

Bacteroides, *Clostridum*, *Lactobacillus* and *Propionibacterium* were obtained in homogenates with inulin supplementation while *Bifidobacterium* counts were similar to the homogenate without supplementation indicating that this population was not significantly influenced by the only presence of the oligosaccharide. Changes on numbers of bacteroides in homogenates without propionibacteria supplementation were pronounced at the second hours of incubation, but the populations remained stable from two to ten hours (Bdis656) or diminished slowly after six hours of incubation (Bfra602). Propionibacteria inhibited the increase on counts of bacteroides at the second hour regardless of the presence of inulin; the effect was more noticeable when the probe Bfra602 was used. A similar behavior to that of bacteroides was observed for the population of clostridia as dairy propionibacteria inhibited the growth of this population in homogenates with or without inulin, being the effect stronger in the last condition. In our experiments, only fluorescent bacteria with rod morphology were counted with Lab158 probe, in order to assess lactobacilli population and exclude enterococci. While the homogenate containing the oligosaccharide supported a fast growth of lactobacilli of almost two logarithmic units in six hours, no significant changes were observed between all the other homogenates after the second hour, even when a slightly reduction of lactobacilli was detected in the homogenate supplemented with only propionibacteria at the end of the incubation. The populations of bifidobacteria showed very similar changes in homogenates without additions and with only prebiotic or propionibacteria during the first six hours. A slow growth of bifidobacteria population was observed in the homogenate containing a combination of inulin and propionibacteria until it reached the same level observed in the other homogenates at six hours of incubation. At the end of the experiment data obtained for this population were similar in all the homogenates. The numbers of *P. acidipropionici* increased slowly until six hours of incubation, but major changes were observed at the end of the experiment in both homogenates inoculated with this bacterium. However, inulin caused the highest increase on propionibacteria at ten hours of incubation, with significant differences related to the homogenate without prebiotic.

3.3. Changes on organic acids production and pH throughout the time

Organic acids production in homogenates with or without inulin, inoculated or not with propionibacteria, was compared throughout the time of incubation (Fig. 2).

The main changes on acids concentrations in the homogenate provided only of endogenous substrates were observed during the first six hours of incubation (Fig. 2A). SCFA and total acids showed similar concentrations in this homogenate during all the experiment as a very little amount of lactic acid was detected throughout the time. The recorded changes on SCFA concentration were close related to acetic acid production due to the little contribution of butyric and propionic acid. In the sample inoculated with propionibacteria, propionic acid concentration increased slightly while other acids diminished its production, and non significant reduction on total acids and SCFA were recorded in this homogenate related to the non inoculated one at 10 h of incubation (Fig. 2B).

The concentrations of lactic and acetic acids increased significantly in the homogenate supplemented with inulin and reached 0.27 and 0.17 mmol/g respectively after 6 h of incubation. No lineal correlation was observed between both acids as 3.5 times more lactic than acetic acid were detected in the second hour of

incubation and only 1.85 times more at the sixth hour (Fig. 2C). In contrast, propionic acid production remains in a constant ratio of 0.13 mmol per mmol of acetic acid during the first 6 h. The homogenate supplemented with inulin and propionibacteria showed a different correlation with 1.49 mmol of propionic acid produced per mmol of acetic acid at the same time of the experiment. This ratio remained constant until the end of the incubation.

Changes on pH values were also recorded throughout the time in each suspension. The pH values for all the fermentation mixtures were in a range of 6.8–6.95 at the beginning of the experiment. They reached mean values of 5.91 and 5.54 in mixtures without inulin (Fig. 2A and B) and 4.66 and 5.06 in suspensions with the prebiotic, without and with propionibacteria addition respectively (Fig. 2C and D) at the end of the experiment. The lowest value was obtained at 6 h of incubation in the suspension supplemented only with inulin.

3.3.1. Principal components and cluster analysis

A Principal Component Analysis (PCA) was performed on the data of concentrations of fermentation products obtained after 10 h of incubation of the cecal homogenates supplemented with inulin (In), propionibacteria (P), both of them (InP) or without supplementation (C). Five acids and ethanol were the variable analyzed for the samples of five independent experiments. In the scatterplot generated after the PCA (Fig. 3), the horizontal axis represented the first principal component (PC1), positively associated with ethanol (83%), formic acid (71%), lactic acid (65%) and butyric acid (59%). This first component was also negatively associated with the production of propionic (67%) and acetic acid (47%).The second principal component (PC2), evidenced the preferential production of lactic acid (56%) and was negatively related to acetic (59%) and butyric acid (58%). The third principal component (PC3), was positively related to butyric acid (49%) and negatively related to formic, propionic and lactic acids (45, 45 and 29% respectively). The three main principal components accounted for 79.2% of the total variance.

Considering the relative positions of the samples on the first three components and the vectors representing ethanol and acids measured (Fig. 3), we have observed that samples of non supplemented homogenates were highlighted above all by the butyric acid content; they also contained ethanol and other acids but in relatively low to medium quantities with a significant variation between samples. Homogenates with added inulin, had the highest production of lactic acid. These samples were also positively related with the production of ethanol and butyric acid, and negatively related with acetic and propionic acids.

In samples with added bacteria, it was observed positive association with acetic and propionic acids and negative with lactic acid and ethanol. Finally, samples with inulin and bacteria showed behavior similar to those that have only bacteria, but with increased production of propionic acid and diminished concentration of acetic acid, stressing the sample InP1 with a maximum value of the propionic and acetic acid.

Cluster analysis was carried out on the basis of fermentation products concentrations. A two-way dendrogram obtained using the squared Euclidian distance and the linkage method of the nearest neighbor is shown in Fig. 4. The maximum and minimum values of Euclidian distances among samples were informed as 0% and 100% of similarity respectively. Samples C2, C3, C4 and C5, that were incubated without supplementations belonged to the same group, with 85% of similarity among them. Samples P1, P3 and P4, from homogenates with propionibacteria, showed 92% of similarity among them while samples InP2, InP3, InP4 and InP5, with 91% of similarity were also grouped. Both groups containing propionibacteria were close related, with 89% of similarity among them. Two samples from homogenates supplemented with propionibacteria (samples P2 and P5) were grouped with samples with combined supplementations due to a lower production of acetic acid and higher of propionic acid related to other replicates. At a limit of 75% of similarity, samples C, P and InP belonged to different branches of the same cluster, designated as branches A, B and C of Cluster 1 respectively, with exceptions of C1 that was deficient in ethanol and butyric and InP1, which accounted for the major amount of propionic and acetic acids. Samples In2, In3, In4 and In5, from homogenates supplemented with inulin were grouped in the Cluster 2. Only one sample from homogenates with inulin (In1) showed low similarity with this cluster due mainly to a higher production of lactic and acetic acids and absence of ethanol.

The second part of the analysis showed the variables clustering. Butyric acid was in low concentration in samples from branch B and C of Cluster 1 and higher in the branch A of Clusters 1 and in Cluster 2. In contrast, lactic acid was in high concentration in Cluster 2, low in branch A of Cluster 1 and absent in branches B and C of the same cluster. Acetic and propionic acids presence was partially associated in the homogenates samples and showed inverse relationship between the branches B and C of Cluster 1.

4. Discussion

Microbial interactions in the intestine are extremely complex taking into account that the final products of carbohydrates fermentation of some species may be reutilized by others modifying the nutritional status of different populations and the final concentration of metabolites, especially SCFA. Acetate is mainly derived from glucose carbon skeleton, but Lajoie et al. [37] showed that the colonic microflora also reduce CO_2 to acetate by using the free H_2 formed as intermediate product during fermentation. Other studies indicated that CH_4, an important product in adult humans harboring significant number of methanogenic organisms [38], is formed from CO_2 and free H_2 with concomitant decrease in acetate production. Propionate is formed by CO_2 fixation pathways, as in *Propionibacterium* and some species of *Bacteroides* [39] or by lactate reduction via lactyl-, acrylyl- and propionyl-CoA as in other *Bacteroides* species and bacteria belonging to the clostridial cluster IX group [19,39]. Butyrate is formed by the classical reaction of acetyl-CoA condensation and further formation of butyril-CoA [20] from glucose in several clostridial clusters or from lactic and acetic acids as in *Eubacterium hallii* [40–42]

The stimulation of intestinal bifidobacteria and lactobacilli in diets supplemented with oligofructose has been considered as the main effect produced by this non-digestible oligosaccharide and supports the claim of the health benefit of oligofructose consumption. However, *Bacteroides* is one of the more saccharolytic genera among the intestinal bacteria and its ability to breakdown inulin and oligofructose of lower degree of polymerization is well known [17]. Therefore, a stimulating effect on the growth of *Bacteroides*, may be expected in these supplemented diets [18]. Mixtures of oligofructose and probiotic strains of bifidobacteria or lactobacilli has been utilized to ensure the improvement of the status of these beneficial populations in the colon, although lactic acid produced by these genera may be, in turns, an energy source for other undesirable bacteria, like strains of clostridia responsible for harmful enzymes activities or toxins production. On the contrary, mixtures of inulin and other SCFA producers as dairy propionibacteria, which are also capable to utilize lactic acid, have not been yet studied as dietary supplement.

In a complex ecosystem, like the intestinal lumen is, the concentration reached by the final products of fermentation may result from constant or sequential formation [43,44]. In order to

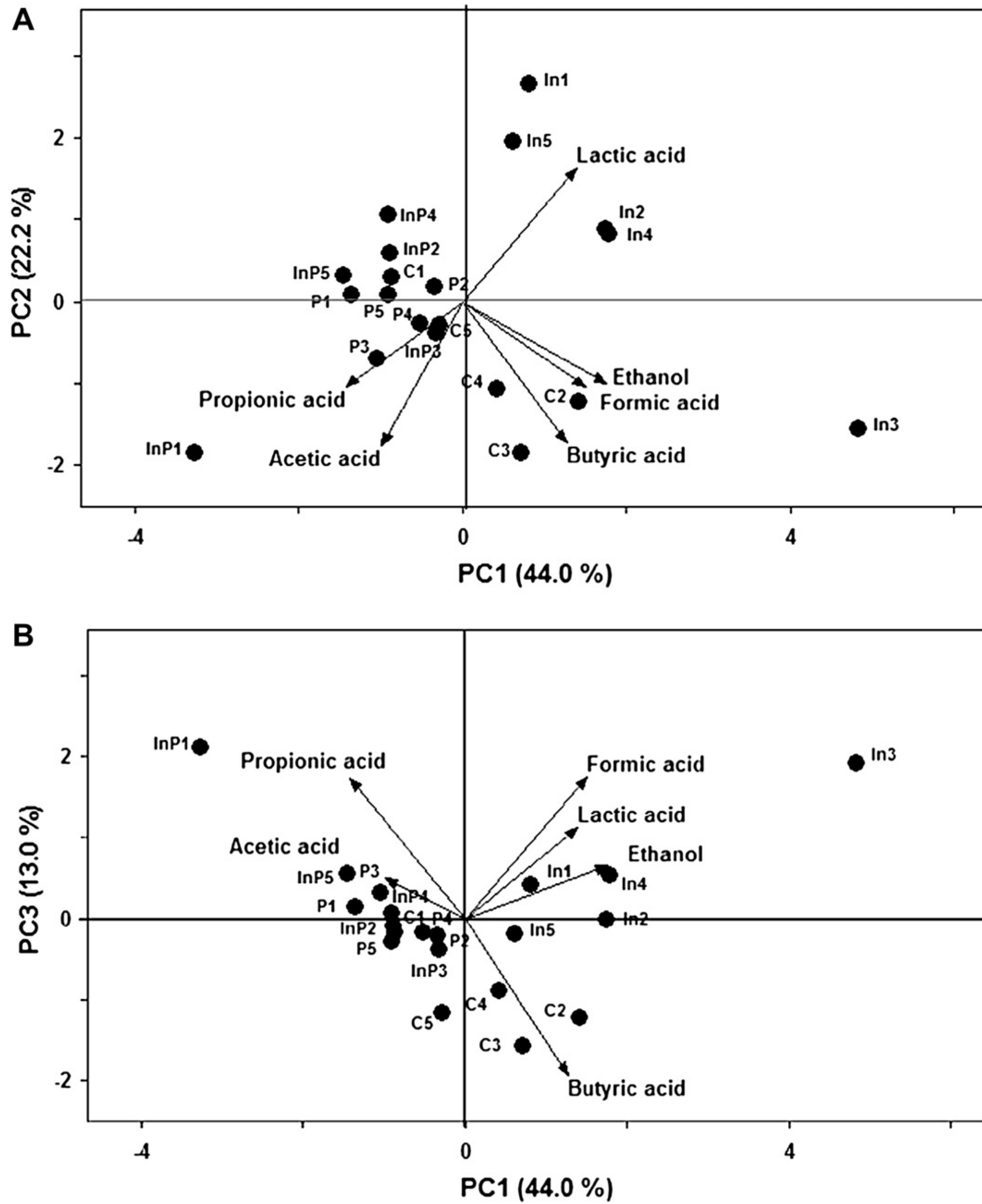

Fig. 3. PCA. Characters C, P, In and InP correspond to samples from homogenates without supplementation, with propionibacteria, inulin and mixed supplementations respectively. Numbers 1–5 in the samples indicate a different replicate. Symbols: (●), samples, (➔), variables.

evaluate this feature in our study, changes on lactic acid, SCFA and ethanol concentrations and on bacterial populations were determined throughout the time of incubation of homogenates with and without supplementation of inulin and propionibacteria. As it is showed in Fig. 1 all the products were detected in some extent since the beginning of the experiment. During the fermentation of endogenous substrates (Fig. 1A), consisting mainly on glucose and minor amount of fructose derived from the diet and breakdown of free mucus (data not shown), the main SCFA detected was acetic acid, which increased rapidly in the first two hours, reached its higher concentration at six hours of incubation and remained constant until the end of the experiment. This acid could be produced by several bacteria like clostridia, bacteroides and bifidobacteria under carbon-limited condition [2] as these populations increased during the incubation of this homogenate (Fig. 2). Even when other SCFA also showed a slightly increase, SCFA ratio changed from 45.4/23.8/30.8 to 61.2/10.6/28.2 for acetic/propionic/butyric acids, indicating a detrimental effect on propionic acid production.

The presence of propionibacteria in this homogenate exerted a strong inhibition on the development of clostridia and negatively influenced on butyric acid production, but a slight increase on the concentration of propionic acid was observed (Figs. 1B and 2). Acetic acid concentration was also diminished although the effect was partially reverted by propionibacteria and bifidobacteria as they are also acetic acid producers. Therefore, acetic/propionic/butyric acids ratio changed from 45.4/23.8/30.8 to 55.9/31.2/12.9. The growth of *Bacteroides fragilis* group (Bfra602 probe) was abolished while *Bacteroides distasoni* group (Bdis656 probe) was inhibited after the second hour of incubation returning to the initial number of cells. A negative effect of low pH values on these populations was discarded as the lowest pH value was obtained with the addition of inulin alone at six hours of incubation when bacteroides and clostridia reached their higher numbers. In the

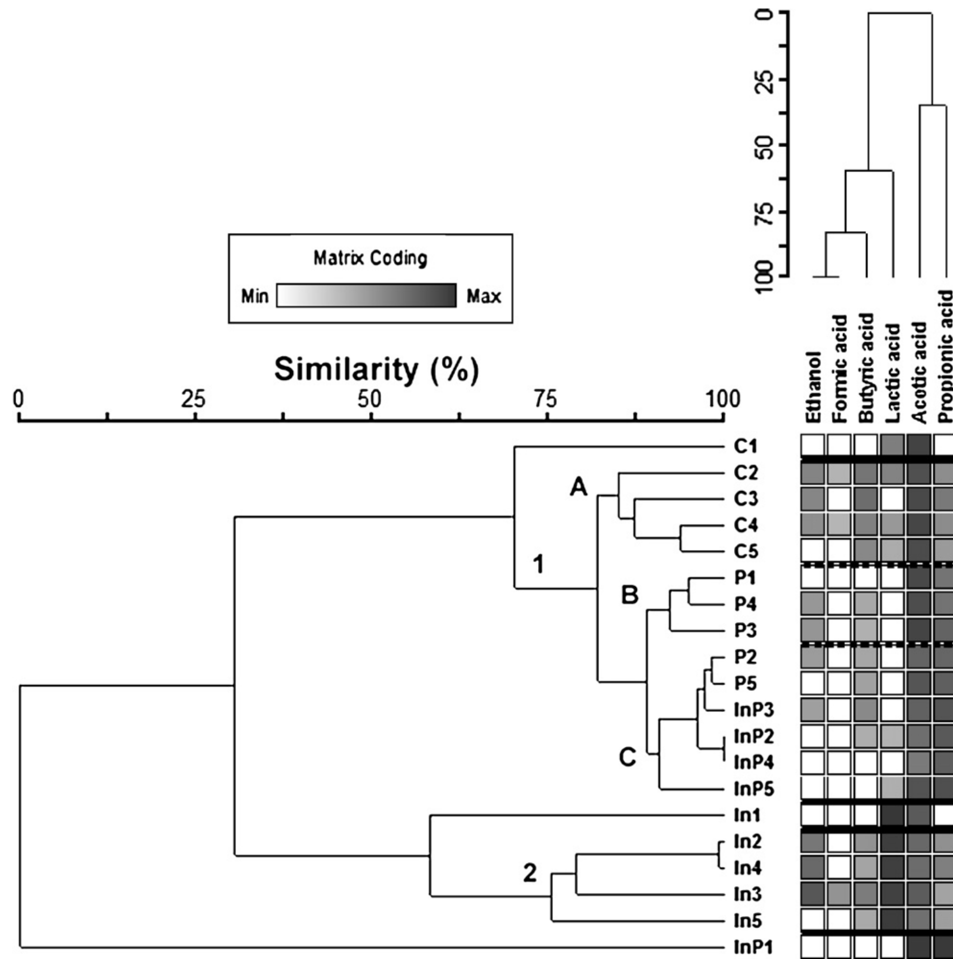

Fig. 4. Two-way dendrogram of the cluster analysis. Euclidean distances are showed as percentage of similarity among samples. Numbers 1 and 2 indicate Clusters and capitals letters A, B and C indicate different branches of Cluster 1 which are also marked with dotted lines in the matrix representation. Colours of matrix elements indicate percentiles of each variable.

intestine, where a very stable microbiota inhabit, complex interactions that exist among the different populations depends on the structure and amount of fermentable carbohydrates, nitrogen sources and production of vitamins and other growth factors. This regulates the total number of microorganisms and the ratio of the different populations in such a way that the increment on one genus may conduces to reduction of one other. The introduction of dairy propionibacteria in high amount (10^9–10^{10} cells/g) in cecal homogenates necessarily conduced to the inhibition of genera that use similar substrates or metabolize sugar by similar pathways. It has been demonstrated that *B. fragilis* metabolizes carbohydrates with production of succinic, propionic and acetic acids in the presence of hemin and lactic and fumaric acids in its absence [19]. In this bacterium propionate is formed from succinate via succinyl-, methylmalonyl- and propionyl-CoA, like in *Propionibacterium*. Therefore, propionic acid detected in homogenates without bacterial supplementation could be due to bacteroides activity while in homogenates supplemented with propionibacteria derived also from the metabolism of the bacteria inoculated. No lactic acid production was detected accordingly with propionibacteria growth, a lactic acid consumer.

During incubation of the homogenate containing inulin, lactic acid production (Fig. 1C) and the development of populations counted with the probe Lab158 (Fig. 2) followed the same profile suggesting that the genus *Lactobacillus* was the main responsible for the increase on lactic and total acids concentrations in this homogenate. Members of *Clostridium* genus could also be involved in both the higher lactic and lower acetic acid production observed related to the control homogenate as this genus produces lactic instead acetic acid when the energy source is in high concentration [2] as occurred at the beginning of the experiment. Other bacteria, such as bifidobacteria, are also lactic acid producers, but in our experiments, its contribution seems to be negligible as inulin did not increase the number of bacteria from this population in the cecal homogenate (Fig. 2). Wang and Gibson [45] observed a stimulant effect of inulin on the growth of bifidobateria in fermentations with human fecal slurries, while Langlands et al. [46] did not found significant changes on planktonic but in mucosal bifidobacteria, both *in vitro* and *in vivo*. Van der Meulen et al. [47] reported that *Bifidobacterium animalis* DN-173010 was not able to growth on Raftilin HP, and also Rossi et al. [48] demonstrated that only eight from fifty-five strains grew on inulin. Therefore, the species composition of the mice cecal content and the high initial density of the *Bifidobacterium* population could determine our results related to the growth of this genus. The detection of ethanol after the second hour and formiate at the end of the incubation period was consistent with the metabolic activity of bifidobacteria (Fig. 1C). No succinic acid was detected, even when it is an important product of bacteroides populations which were also in higher number, suggesting the assimilation of this organic acid by other components of the microbiota. Rossi et al. [48] reported that butyrate is the main product obtained from inulin in batch cultures inoculated with

human fecal slurries. However, fecal cultures were carried out in an artificial complex medium and incubated during 24 h probably allowing the selection of specific butyric acid producer populations from the microbiota as was reported in other *in vivo* studies. In our experiments butyrate production was not stimulated by inulin. On the contrary, SCFA rates at ten hours of incubation of this homogenate were 65.5/15.7/18.8, suggesting a remarkable negative effect on both propionic and butyric acid production.

The simultaneous supplementation with inulin and propionibacteria limited very strongly the growth of bacteroides and clostridia, which remained in constant number after the second hour of the experiment. On the other hand, propionibacteria supplementation counteracted the stimulatory effect of inulin on the growth of lactobacilli. A change on the growth kinetic was observed in the bifidobacteria population which reached a number similar to that observed in other homogenates after a slow growth of six hours. This suggested that a competition for inulin breakdown products could have been established between bifidobacteria and propionibacteria during the first hours of incubation of this homogenate as growth delay was detected only when inulin and propionibacteria were added together. A further adaptation of both populations was observed as bifidobacteria reached a final count as higher as the registered in other homogenates.

A shift on the global metabolic activity of the cecal microbiota in the homogenate supplemented with inulin and propionibacteria was evidenced. The negative effect on propionic and butyric acid production observed during inulin supplementation was partially reverted as SCFA rates were 46.1/44.0/9.9. The ratio propionic acid/acetic acid reached a constant value throughout the time of 1.49, compared to the ratio of 0.13 obtained in the homogenate supplemented with only inulin. An increase on this ratio is usually considered a favorable change taking into account that acetic acid contributes to the cholesterol synthesis in the liver while propionic acid exerts the opposite effect [49]. The beneficial effect of feeding mice with a dairy hyperlipemic diet containing propionibacteria on triglycerides and cholesterol concentrations has been previously demonstrated [22], and the effect has been attributed mainly to propionic acid production from the fermentation of lactose and intestinal carbohydrates residues.

In our experiments we showed that inulin increased lactic acid concentration during the ten hours of incubation of the cecal homogenates of mice, according to an expected increase on lactobacilli population, and reduced the SCFA concentration regardless acetic acid increase. When propionibacteria were included in homogenates, a remarkable increase on propionic acid, and reduction of lactic acid concentration was observed in the homogenate supplemented with carbohydrate (Fig. 1D), while a little increase on propionic acid was detected without carbohydrate addition (Fig. 1B). These results allowed conclude that fermentation of inulin did not improve by itself total SCFA production but acetic acid production. In contrast, the incorporation of propionibacteria in diets may be proposed as a tool to increase the propionic acid concentration in diets containing inulin.

The PCA confirmed that lactic and propionic acid productions were negatively related (Fig. 3a and b), suggesting the partial consumption of lactic acid to produce propionic acid in homogenates containing *P. acidipropionici* CRL1198. This acid production was also opposite to butyric and ethanol production suggesting the inhibition of microbial populations responsible for the production of these compounds at expense of lactic acid. Acetic and propionic acids showed low correlation as acetic acid, the main product found in the intestinal contents, derived from substrates fermentation of several populations (Fig. 4).

Clusters analysis demonstrated that samples from homogenates with propionibacteria belong to the same cluster independently of the carbohydrate supplementation in the InP samples. The fermentation products measured in samples P and InP were similar even when lower acetic and higher propionic acids production were observed during the mixed supplementation.

Our results allowed conclude that dairy propionibacteria, either alone or in combination with inulin in the intestinal environment, may contribute to the control of anaerobic genera like *Bacteroides* and *Clostridium* which include species that are associated with several human diseases [50–54] and risk factors of cancer [55]. Inulin supplementation may improve lactobacilli development and acetic acid production. However, total SCFA concentration is not enhanced. On the contrary, the combination of inulin with propionibacteria seems to be a useful tool to ensure SCFA production, especially propionic acid, with known benefits on human health.

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