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Influence of lactose and lactate on growth and β-galactosidase activity of potential probiotic *Propionibacterium acidipropionici*

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ABSTRACT

Dairy propionibacteria are microorganisms of interest for their role as starters in cheese technology and as well as their functions as probiotics. Previous studies have demonstrated that *Propionibacterium acidipropionici* metabolize lactose by a β-galactosidase that resists the gastrointestinal transit and the manufacture of a Swiss-type cheese, so that could be considered for their inclusion in a probiotic product assigned to intolerant individuals. In the present work we studied the effect of the sequential addition of lactose and lactate as first or second energy sources on the growth and β-galactosidase activity of *P. acidipropionici Q4*. The highest β-galactosidase activity was observed in a medium containing only lactate whereas higher final biomass was obtained in a medium with lactose. When lactate was used by this strain as a second energy source, a marked increase of the intracellular pyruvate level was observed, followed by lactate consumption and increase of specific β-galactosidase activity whereas lactose consumption became negligible. On the contrary, when lactose was provided as second energy source, lactic acid stopped to be metabolized, a decrease of the intracellular pyruvate concentration was observed and β-galactosidase activity sharply returned to a value that resembled the observed during the growth on lactose alone. Results suggest that the relative concentration of each substrate in the culture medium and the intracellular pyruvate level were decisive for both the choice of the energetic substrate and the β-galactosidase activity in propionibacteria. This information should be useful to decide the most appropriate vehicle to deliver propionibacteria to the host in order to obtain the highest β-galactosidase activity.

1. Introduction

The ability of microorganisms to ferment lactose of milk is an important technological property. Hydrolysis of the sugar that confers taste, texture and nutritional value to milk and derivatives is carried out by the enzymes β-D-galactosidase (EC 3.2.1.23) and/or phospho-β-D-galactosidase (EC 3.2.1.85). These enzymes have been described in different organisms such as bacteria, yeasts and moulds [1–4]. Besides their technological importance, the pure enzymes or the viable microorganisms containing them have been used to alleviate intestinal disorders such as lactose intolerance [5–7]. This phenomenon is worldwide spread among adult population and has been treated successfully by the incorporation of microorganisms, mainly lactobacilli or bifidobacteria, in dairy products as a source of β-galactosidase for the intraintestinal hydrolysis of lactose or the modulation of colonic microbiota [8–11]. In this sense, many studies have focused on the metabolism of lactose by potentially probiotic microorganisms.

Dairy propionibacteria are microorganisms of interest since they are extensively used for manufacture of Swiss-type cheeses, biological production of propionic acid and vitamin B_{12} [12,13]. In recent years, many potential probiotic properties have also been reported for these microorganisms [14–19]. Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confers a health benefit on the host" [20]. In this sense, potential beneficial effects of propionibacteria on health have been related to their ability to produce vitamins, bacteriocins [21], the stimulation of growth of other colonic bacteria like bifidobacteria [22,23], the improvement of the intestinal microbiota composition and their metabolic activities [14,15,24], as well as their immunomodulating, antimutagenic and anticarcinogenic properties [25–29].

In previous studies, we demonstrated that strains of *Propionibacterium acidipropionici* hydrolyze lactose by utilizing β-galactosidase as they do not posses phospho-β-D-galactosidase activity. Besides, the main biochemical characteristics of the enzyme in this

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species were described for the first time [30]. However, it is thought that to digest lactose in vivo in the intestine the selected microorganisms and their β-galactosidase must be able to survive the harsh environment conditions of the gastrointestinal tract and the technological processing of the product that include them. In this respect, we demonstrated that the β-galactosidase of *P. acidipropionici* resist the adverse gastrointestinal conditions and the manufacture of a Swiss type cheese so that the strains could be considered for the manufacture of a probiotic product assigned to intolerant individuals [24,31]. Probiotic cheeses for delivery of beneficial propionibacteria may represent an alternative to other conventional fermented dairy products such as yogurt and fermented milks. Development of cheeses as a vehicle for probiotics also represents a research topic of growing interest for dairy industry [32] and an enterprise with few antecedents in Argentina [33,34].

In the present study, we mainly compared the β-galactosidase activity of a potential probiotic strain of *P. acidipropionici* growing on lactose, lactate and their combination as energy sources since scarce information is available about the influence of different combinations of energy sources on growth and enzyme activity of this species.

2. Materials and methods

2.1. Microorganism and culture conditions

The strain Q4 used in this study was isolated in our laboratory from a Swiss-type cheese made in Argentina. It was identified as *P. acidipropionici* by biochemical tests described in the Bergey's. Manual of Systematic Bacteriology and the16S rDNA sequence; and was physiologically characterized in previous studies [15,31,35–37]. The strain was stored at −20 °C in 10 % (w/v) reconstituted skim milk (RSM) containing 5 g/L yeast extract and was activated by three successively transfers every 24 h in a broth medium with the composition of YEL [38] in which sodium lactate was replaced by 0.1 % (w/v) glucose .

2.2. Cultures in media with lactose and lactate as energy sources

Active cultures of propionibacteria were centrifuged, washed and suspended to the original volume in 0.9% NaCl and incubated at 37 °C during 3 h to deplete cells of intracellular reserves. This cell suspension was inoculated at the rate of 2% (v/v) in the basal broth described above without glucose, supplemented with 1% (w/v) lactose (30 mM) or sodium DL-lactate (110 mM of equal amounts of L-(+) and D-(−) isomers) and incubated at 37 °C without shaking during 50 h.

In another trial, cultures on lactose or lactate were incubated as mentioned and after 24 h they were fractioned into two aliquots. One of them was supplemented with a second energy source, lactate or lactose respectively, in final concentration of 1% and incubated up to the stationary phase of growth (28–50 h according to the condition).

Samples were taken at different time intervals for determinations of biomass, β-galactosidase activity, substrates consumption and intracellular concentrations of pyruvate.

2.3. Determination of growth and substrates consumption

Increase in biomass was determined turbidimetrically by optical density measurements at 560 nm (Spectronic 2000, Bausch & Lomb Spectrophotometer). Concentrations of substrates present in samples supernatants were determined spectrophotometrically by enzymatic methods. Lactose-Galactose kit (Cat. No. 176303 available from Boehringer-Mannheim, Germany) for lactose determination and D/L Lactic acid kit (Cat. No. 1112.821; Boehringer-Mannheim, Germany) for assess L-(+) and D-(−) lactate concentrations were used. Reactions were performed according to the manufacturer's instructions.

2.4. Determination of β-galactosidase activitiy

Cells were centrifuged (10,000 × *g*, 10 min, 4 °C), washed twice with 50 mM KH_2PO_4–Na_2HPO_4 buffer, pH 7.0, resuspended in buffer to an $O.D._{560nm}$ of 0.5 units and then concentrated 20 fold (approximately 1×10^{10} cells mL^{-1} and 3.85 ± 0.16 mg of dry weight). The β-galactosidase activity was determined by measuring the hydrolysis rate of the synthetic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) with a colorimetric method. The enzymatic reaction was carried out with 200 μL of cell suspension, 0.836 mM ONPG and 50 mM KH_2PO_4–Na_2HPO_4 buffer, pH 7 in a final volume of 1 mL. The mixtures were incubated for 15 min at 37 °C and the reactions stopped with 800 μL of 0.5 M Na_2CO_3. Reaction mixtures were clarified by centrifugation (5000 × g, 10 min) before determining the absorbance at 440 nm. The enzymatic activity was expressed as nanomols of *o*-nitrophenol (ONP) liberated per minute per milliliter of the suspension of 1×10^{10} cell mL^{-1} [31].

2.5. Determination of intracellular pyruvate concentrations

The concentration of intracellular pyruvate was determined enzimatically at 24 h of growth in media with lactose or lactate and 2 and 24 h after the addition of the second energy source (lactate to the culture with lactose and lactose to the culture grown on lactate) as described by Czok and Lamprecht [39]. Aliquots of each culture were diluted in broth media to obtain 5 mL of culture at OD_{560} equal to 1. They were centrifuged (10 min, 4 °C, 10,000 × *g*) washed with sterile water and resuspended in 5 mL of $HClO_4$ 0.6 N, kept on ice during 15 min in order to broke cells and precipitate proteins and finally centrifuged 5 min at 10,000 × g. The supernatant was neutralized with KOH 2N and centrifuged again. The reaction mixture for pyruvate detection contained: 0.8 mL of sample, 1.2 mL of triethanolamine buffer, 0.5 M, pH 7.6 with 5 mM EDTA, 0.04 mL of 6 mM NADH, and 0.02 mL lactate dehydrogenase (LDH). Extinction was read at 365 nm and the pyruvate levels initially calculated in μmols mL^{-1} of neutral extract [39] were transformed into intracellular pyruvate concentrations as reported by Crow [40].

2.6. Statistical analysis

The results informed are the average of the data obtained from three independent trials of each experiment. Significant differences between means were determined by Tukey's test after analysis of variance (ANOVA) with Minitab Statistic Program, release 14 for Windows. A *P* value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Influence of the energy source on growth and β-galactosidase activity

In the present study, the influence of lactose and lactate, two carbohydrates that could be present together in dairy products or the intestine, on growth and β-galactosidase activity of *P. acidipropionici* Q4 was determined in a complex medium.

Initial concentrations of lactose or DL-lactate in the culture media were 1% (w/v) that represent 30 mM of lactose and 110 mM of lactate respectively and were in the range of those normally encountered in dairy products and the intestine [41,42] . These concentrations and the ratio between substrates (1 lactose: 4 lactate) were selected by

taking into account that fermentation of 1 mol of lactose in a dairy product or the intestine leads to almost 4 mols of lactate. If we consider (as an extreme case) that no lactose is digested in the intestine, all lactose should be fermented to lactate by the intestinal microbiota and this would represent the initial substrate for fermentation by propionibacteria (and other intestinal bacteria).

As shown in Fig. 1 biomass obtained at the end of growth was significantly higher in the culture supplemented with 30 mM lactose, as final $O.D._{560nm}$ was up to 2.5 times higher than that obtained in the presence of 110 mM sodium DL-lactate ($P < 0.05$). Stationary phase of growth was reached between 28 and 32 h of incubation in the presence of lactate and at 50 h when the substrate was lactose. On the contrary, higher β-galactosidase activity was observed in the cell suspensions grown in medium supplemented with sodium lactate than in cells developing on lactose ($P < 0.05$), in agreement with previous reports for cultures exponentially growing [30]. In this sense, Hartley and Vedamuthu [43] also reported a slightly higher β-gal activity of strains of *Propionibacterium shermani* exposed to sodium lactate than lactose.

P. acidipropionici Q4 showed a basal level of β-galactosidase of approximately 30 nanomols min^{-1} mL^{-1} of cells suspension (7.8 nanomols min^{-1} mg^{-1} of cells) that increased during exponential growth on both lactose and lactate reaching a maximum activity that was 3 and 7 times higher than the basal value, respectively (Fig. 1).

It is known that *Propionibacterium* can obtain energy for growth from glucose, galactose or lactose (if posses β-galactosidase) or lactate, but biomass yields are different depending on the carbon source used. The lactate pathway generates ATP by conversion of acetil-CoA to acetate and reduction of fumarate to succinate. The metabolism of glucose also yields energy by phosphorylation at substrate level during conversion of PEP to piruvate. In consequence, according to the theoretical fermentation balances it could be expected in the culture growing on lactose a higher ATP production that was used for biomass production [44].

3.2. Effect of lactate and lactose as second energy sources on growth and β-galactosidase activity

When lactate was added in the middle of the exponential phase to the culture that was growing on lactose, an immediate increase of β-galactosidase specific activity was observed ($P < 0.05$), whereas growth stopped with a final biomass similar to that of the culture grown on lactate (Fig. 2A). This pronounced increase of activity was probably due to the role of lactate as enzyme activator as was previously reported [30].

On the contrary, when propionibacteria were growing on lactate, the addition of lactose to the 24 h culture, changed the energy source being used (Fig. 2B). The high β-galactosidase activity observed at this time in the culture growing on lactate, and the high concentration of lactose added seemed to favor the disaccharide consumption, since biomass obtained at the end of incubation resembled that of cultures with lactose as the only energy source. However, during the following hours the enzyme activity decreased and at the end of the experiment it was slightly lower than the observed in cultures on lactose alone (Fig. 2B).

3.3. Effect of substrates and pyruvate concentrations on the preferential utilization of the second energy source

Since a relationship between substrates concentrations, growth and β-galactosidase activity seems to be present, the consumption of lactate and lactose, the intracellular pyruvate levels and the

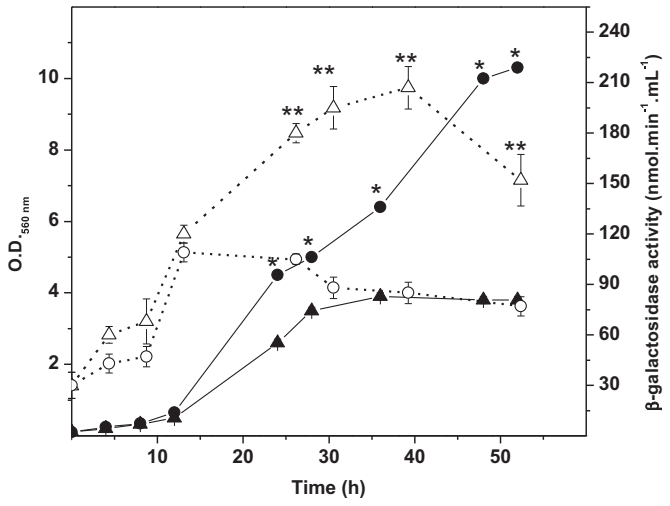

Fig. 1. Growth (closed symbols) and β-galactosidase activity (open symbols) of *Propionibacterium acidipropionici* Q4 in complex medium containing lactose (●, ○) or sodium DL-lactate (▲, △) as energy sources. *The asterisk indicates significant differences between growth curves ($P < 0.05$). **Indicates significant differences between enzyme activity curves ($P < 0.05$).

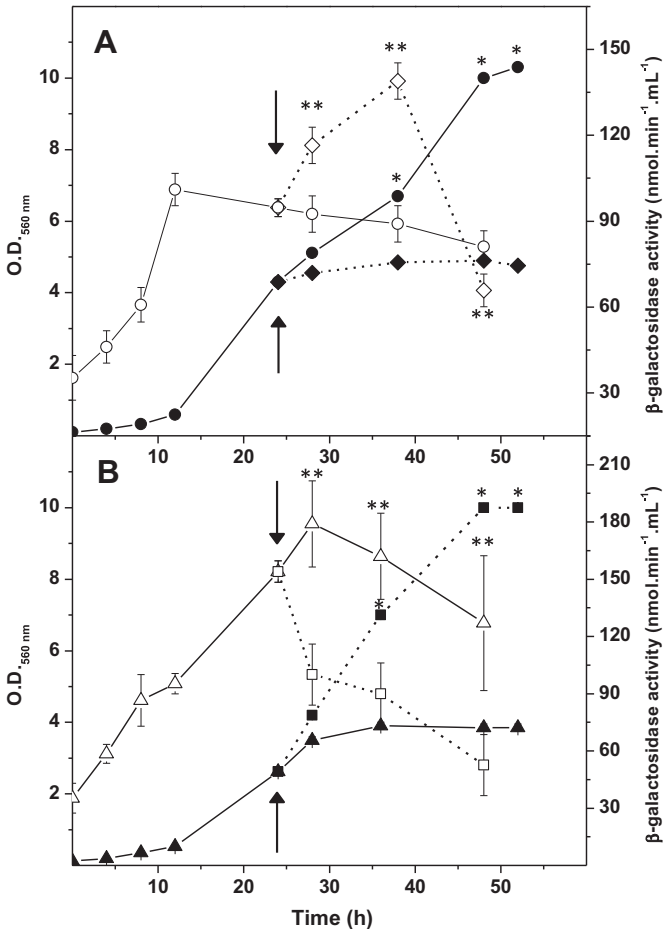

Fig. 2. Growth and β-galactosidase activity of *P. acidipropionici* Q4 in complex medium containing initially 30 mM lactose (A) or 110 mM DL-lactate (B), followed by the addition of lactate or lactose respectively in the middle of exponential phase of growth (black arrows). Symbols: (●) growth and (○) activity on lactose; (◆) growth and (◇) activity on lactate as second substrate; (▲) growth and (△) activity on DL-lactate; (■) growth and (□) activity on lactose as second energy source. *The asterisk indicates significant differences between growth curves ($P < 0.05$). **Indicates significant differences between enzyme activity curves ($P < 0.05$).

β-galactosidase activity before and after the addition of the complimentary energy source were determined.

When the culture growing on lactose was supplemented with 110 mM of DL-lactate (55 mM of each isomer), the concentration of the new energy source was at this moment higher than that of the residual lactose (12.58 mM) and under this condition, *P. acidipropionici* Q4 metabolized preferentially lactic acid during the following hours (Fig. 3A). The lactose consumption was high in the first 24 h (17 mmols L^{-1}) and negligible during the 24 h that followed the addition of lactate (2.6 mmols L^{-1}).

Growth, since addition of lactate, took place at expense of the L(+) isomer, remaining in the culture medium 41.7% of lactic acid, mainly D(−)-lactate, and 34.4% of lactose at 48 h of incubation. It has been reported previously that the preferential utilization of L(+) lactate isomer by strains of *Propionibacterium freudenreichii* susbsp *shermani* results from the inhibitory effect of pyruvate on D(−)-LDH, enzyme that metabolize the D(−) isomer of lactic acid [40]. In our experiments, a great increase in the intracellular pyruvate concentration was observed two hours after lactate addition (26 h of the culture), reaching a level of almost 60 mM that was enough to inhibit the activity of D(−)-LDH [40]. It has been observed in *Aggregatibacter actinomycetemcomitans*, a gram negative opportunistic pathogen of the oral cavity, that although higher cell yields are achieved when catabolizing glucose, L(+)-lactate is preferentially utilized [45]. Interestingly, L(+)-lactate addition to a chemically defined medium inhibited uptake of glucose by *A. actinomycetemcomitans* and high levels of intracellular pyruvate were critical for this L(+)-lactate preference [46]. The mechanism proposed for this microorganism, involves a lactate-dehydrogenase which is not feedback inhibited by pyruvate. This lack of feedback inhibition allows intracellular pyruvate to rise to levels sufficient to inhibit glucose uptake by a process called phosphotransferase (PTS) substrate exclusion. Although the PTS enzyme affected by pyruvate is not known it has been hypothesized that inhibition occurs at the first step of PTS transport, phosphorilation of protein EI by phosphoenolpyruvate (PEP) [46]. Recently, the complete genome of *P. freudenreichii* was sequenced and some components of mannose PTS system were found [47]. However, no lactose PTS system was described yet in *Propionibacterium* and lactose uptake seems to be driven by a permease system [30,43,47].

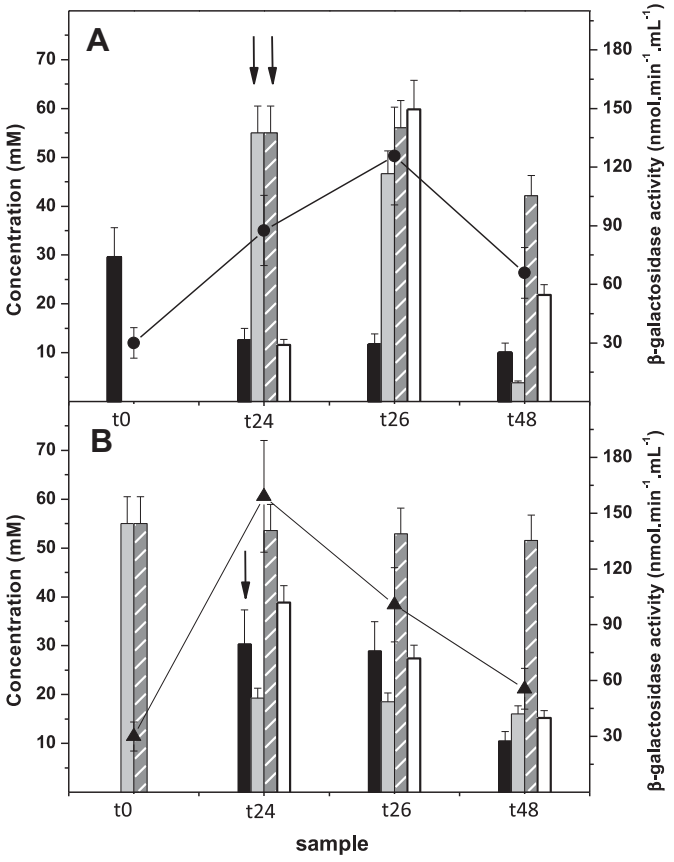

Fig. 3. Substrates, intracellular concentration of pyruvate and β-galactosidase activity of *Propionibacterium acidipropionici* Q4 in complex medium containing initially 30 mM lactose (A) or 110 mM DL-lactate (B), followed by the addition of lactate or lactose respectively in the middle of exponential phase of growth (black arrows). Symbols: (■) lactose; (▨) L(+)-lactate;(▨) D(−)-lactate; (□) pyruvate; (●, ▲) β-galactosidase activity. Data are presented as mean ± SD.

On the other hand, in the culture growing initially at expense of lactate (Fig. 3B), 33.8% (approx. 37 mM) of total lactic acid was consumed at the moment of lactose addition, corresponding 65% to the L(+) isomer and 2,6 % to the D(−) isomer. During the two subsequent hours of lactose addition, the consumptions of this sugar and lactate were low since the lactose concentration diminished in 1.43 mM and L(+)-lactate in 0.75 mM, whereas intracellular pyruvate sharply decreased in 11.4 mM and continued descending up to the end of incubation.

Although the total lactate concentration present in the culture when lactose was added (almost 73 mM) was higher than this sugar (30.33 mM), the concentration of intracellular pyruvate present (almost 40 mM) seemed to be high enough to inhibit the activity of D(−)-LDH. In consequence, after the transition period of 2 h previously described, preferential consumption of lactose was observed due to the higher amount of this substrate related to the residual L(+)-lactate (18.6 mM) and the lower pyruvate concentration which continued descending up to the stationary phase of culture. As a result of the metabolism of lactose by the Embden-Meyerhof pathway a higher production of biomass was evidenced due to the higher ATP yield obtained from this energy source. After 48 h of incubation, consumptions of 64.2% of the lactose added and 38.6% of the initial lactate were registered.

Lactic acid was not significantly metabolized during the active consumption of lactose, so that β-galactosidase activation decreased and the values of the enzyme activity were similar to those observed during the growth on lactose alone (Figs. 2B and 3B). The decrease of pyruvate after supplementation with lactose, paralleled the decrease of β-galactosidase (the reduction of 1 mM of the concentration of intracellular pyruvate diminished 4.37 times the enzyme activity), which support the relationship among the β-galactosidase activity and the intracellular lactate metabolism. However, the steps involved in the regulation of the enzyme synthesis during growth in media containing lactate remains to be elucidated.

The ability of microorganisms to sense their environments and choose the substrate to be consumed, such as lactate despite its inferiority as a carbon and energy source, has many advantages as it represents a survival strategy that allows microorganisms to exclude themselves from the competition for limited carbon sources that take place in their natural environment. This strategy is extensively used by the classical propionibacteria and evidenced by their profuse development during the maturation of Swiss-type cheeses at expense of the lactic acid produced by streptococci and lactobacilli of the starter culture.

According to the main results of our paper, lactic acid should increase enzyme activity to a greater extent than lactose so that propionibacteria should be developed in the presence of this carbon source in order to obtain a high β-galactosidase activity for rapid hydrolysis of lactose. In this respect, propionibacteria should have more clinical than technological relevance, since the usage of

these microorganisms to decrease the lactose levels of dairy products would have less importance than their contribution to the intestinal metabolism of dietary lactose by increasing the hydrolysis in the small bowel. Besides, it could be expected that propionibacteria contribute to lactose metabolism by producing SCFA from the non-digested lactose that arrives to the colon, which in turn should prevent the osmotic diarrhea induced by undigested carbohydrates [11,48].

In consequence, propionibacteria intended to contribute to the metabolism of lactose in the intestine should be included in a fermented product that already contains a high level of lactic acid like a swiss type cheese [49] in order to deliver bacteria and their enzyme in a high competent state to the host.

In a previous study we have demonstrated that *P. acidipropionici* CRL 1198 and its β-galactosidase arrived active to the duodenum and caecum of mice and that intestinal β-galactosidase activity increased during the consumption of an experimental cheese elaborated with propionibacteria, in a higher level than the observed during consumption of milk supplemented with the same bacteria [24]. Besides, we have observed that mice cecal slurries supplemented with propionibacteria, as well as continuous feeding with *P. acidipropionici* modified colonic fermentation by increasing the SCFA production particularly propionate concentration [24,50].

In light of the expression of β-galactosidase of propionibacteria in the presence of lactic acid (that could be provided by a fermented product or the bacterial metabolism in the intestine) further researches are encouraged about the persistence of a high enzyme activity in the bowel for digestion of dietary lactose.

3.4. Conclusions

The information obtained from our research allows predicting the behavior of propionibacteria in different environments in which both lactose and lactic acid would be found in various relative concentrations. In a dairy fluid product, lactose content is high whereas lactate is in a lower concentration. On the contrary, during cheese maturation, the lactose content of the curd is negligible whereas lactate concentration is high. Finally, the metabolism of carbohydrates by the intestinal endogenous microbiota also conduces to the production of lactic acid that propionibacteria are able to consume whereas lactose could be present in different concentrations, from negligible to high, depending on age, lifestyle and intestinal lactase activity of the consumers. In this context the present results could be of industrial relevance since they provide valuable information for selecting the more suitable technology that ensures a high β-galactosidase activity of propionibacteria included into a product aimed to contribute to the intestinal metabolism of lactose.

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