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Influence of Prebiotic Ingredients on the Growth Kinetics and Bacteriocin Production of *Lactococcus lactis*

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Prebiotic ingredients are increasingly added to food preparations containing probiotics in order to enhance probiotic survival and growth. Bacteriocins produced by *Lactococcus lactis* (nisin) have great advantages as a food additive, such as heat stability, nontoxicity and sensitivity to digestive proteases. In this context, the aim of this study was to evaluate the influence of prebiotic ingredients on the fermentation kinetics and antimicrobial activity by *Lactococcus lactis* subsp. *lactis* CECT 4434 against *Lactobacillus sakei*. *Lc. lactis* was cultivated in MRS medium (Man, Rogosa and Sharpe) supplemented with or without (control) fructooligosacharides (MRS+FOS), polydextrose (MRS+PD) and inulin (MRS+IN). All cultivations were carried out in shaken flasks at 30 °C with agitation speed of 100 rpm. Samples were collected for analyses (biomass, specific growth rate, generation time and antimicrobial activity) every two hours during the 48 h of cultivation. The exponential growth phase of *Lc. lactis* subsp. *lactis* CECT 4434 occurred at intervals of 2-8 h for all runs (MRS, MRS+FOS, MRS+PD e MRS+IN). The use of all prebiotic ingredients increased cell biomass, specific growth rate and consequently decreased generation time when compared with control (only MRS). Antimicrobial activity of nisin produced by *Lc. lactis* subsp. *lactis* CECT 4434 was detected against *Lactobacillus sakei* strain.

1. Introduction

Recently, the influence of prebiotic ingredients over the cellular growth of lactic acid bacteria (LAB) has sparked an increased interest due to their properties as a functional food and their beneficial effects to the health and well-being of those ingesting them.

Functional dairy products contain probiotic microorganisms belonging to the *Lactobacillus, Bifidobacterium, Streptococcus* and *Saccharomyces* genera. These products have been historically defined as food containing live microorganisms that improve the host's health through the improvement of the microbiotic balance in the human intestinal tract (Tamine, Saarela, Sondergaard, Mistry & Shad, 2005). By definition, probiotics, including some species of *Lactococcus*, are known as living microorganisms which, when used in adequate quantities, provide benefits to the host's health due to being capable of surviving the trip through the upper digestive tract, adhering to intestinal cells and contributing to intestinal balance (FAO/WHO, 2002; Martinez et al., 2013).

Prebiotics are non-digestible hydrocarbons that, through an absorption process, resist hydrolysis in the upper gastrointestinal tract of humans (Mattila-Sandholm et al., 2002; Apolinário et al., 2014). When consumed in adequate amounts, these ingredients reach the colon still intact and improve the modulation and composition of the intestinal microbiota, providing benefits to the host's health and, therefore, of the users (Saad et al., 2013; Roberfroid, 2007; Roberfroid et al., 2010).

Rastall (2010) studied the applications and the industrialization of prebiotics and reported that, although several hydrocarbons are marketed as prebiotics throughout the world, the most prominent ones are the

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inulin, the fructooligosacharides (FOS) and the galactooligosacharides (GOS). Those are, therefore, the most widely studied hydrocarbons in human clinical tests.
Previous studies have assessed the influence of several prebiotics in the growth and acidification of pure probiotic cultures and co-cultures in skimmed fermented milk. These studies reported that the addition of inulin significantly reduces the fermentation time and increases the viability of probiotic bacteria (Oliveira et al., 2011).
In order to achieve a better understanding of the functional activity of prebiotics over probiotic bacteria, it is necessary to develop better symbiotic preparations. The success in the establishment of the necessary symbiotic condition is closely related to the compatibility between the chosen components, that is to say, between the prebiotic ingredients and the probiotic bacteria (Mei et al., 2011; Pranckute et al., 2014, Muñoz et al., 2012). The authors suggest that strains of *Lactobacillus* sp. and *Lactococcus* sp. may be useful as probiotic bacteria when used together with prebiotics (palatinose, inulin and α-cyclodextrin) so as to create a symbiotic development, which could control not only the grown of beneficial bacteria in the gastrointestinal tract, but also their antibacterial activity.
Under the light of this information, this study aims at assessing the influence of several prebiotic ingredients FOS, polydextrose and inulin over the cellular growth of the *Lactococcus lactis* CECT 4434 bacterial strain and evaluate the growth kinetics parameters (μ_{max} e t_g) when cultivated in MRS medium (Man, Rogosa and Sharpe) supplemented or not (control) with fructooligossacharides (MRS+FOS), polydextrose (MRS+PD) and inulin (MRS+IN). The antimicrobial capacity of the bacteriocin produced by *Lc. lactis* was also evaluation in contrast to the bioindicator strain *Lactobacillus sakei.*

2. Material and methodology

2.1 Microbial culture and growth medium

The strain of *Lactococcus lactis* subsp. *lactis* CECT 4434, obtained from the Spanish Cultivation Collection (CECT), was cultivated in MRS medium (Man, Rogosa and Sharpe) supplemented or not (control) with fructooligosacharides (MRS+FOS), polydextrose (MRS+PD) and inulin (MRS+IN). The MRS growth medium contains 20 g/L of polydextrose. For the formulation of the MRS+FOS, MRS+PD and MRS+IN, the dextrose polysacharide was substituted respectively by 20 g/L of fructooligosacharides, polydextrose and inulin.

2.2 Inoculum preparation and cultivation conditions

The inoculum was prepared with the addition of 100 µL of the stock culture in a 250 mL Erlenmeyer flask containing 100 mL of MRS supplemented with one of the hydrocarbons. The flasks were then incubated in a shaker at 100 rpm and 30°C until a cellular concentration of 0.8 – 0.9 D.O. units at 600 nm.
The cultivation of *Lc*. *lactis* CECT 4434 was conducted with the addition of 10% (v/v) of the inoculum in 250 mL Erlenmeyer flasks containing 100 mL of MRS supplemented individually with the prebiotic ingredient under analysis. The flasks were then incubated at a shaker at 100 rpm and 30°C for 48 hours. Samples were collected every 2 hours for a period of 12 hours and then at intervals of 12 hours, with a total cultivation time of 48 hours.

2.3 Analysis Procedures

In the monitoring process of the cellular growth, the relationship between D.O. (600 nm) and dry mass (mL) was observed after filtration of the growth medium through a 0.22 µm membrane (Millipore). Through this relationship, a calibration curve was obtained, represented by the following equation (1)

$$y = 2.0076 * x + 0.00182 \quad (1)$$

2.4 Growth Kinetics

The growth kinetics for *Lc*. *lactis* CECT 4434 was investigated during the fermentation, both in the absence of prebiotics (control) and in their presence. The maximum specific speed for the growth (μ_{max}) was calculated during the exponential growth phase through the following equation (2).

$$\mu max = \frac{1}{(t_2 - t_1)} \ln \frac{X_2}{X_1} \quad (2)$$

with X_2 and X_1 representing respectively the dry mass values, in (g/L), at t_1 and t_2.

The generation time was determined through equation (3).

$$t_g = \ln 2 / \mu max \quad (3)$$

2.5 Determination of antimicrobial activity

For detection of bacteriocin activity, samples were centrifuged at 16,000 *g* at 4 °C for 10 minutes. The pH of the supernatant was neutralized to 6.0 – 6.5 using 1 M NaOH in order to eliminate the action of organic acids. In addition, the supernatant was submitted to 80 °C for 10 minutes to eliminate possible proteases. After treatment of the supernatant, it was tested against the strains *L. sakei* ATCC 15521 to evaluate its antimicrobial activity. This test was performed by the agar diffusion assay, in which 10 µL of the indicator strain were transferred to a Petri dish containing 15 mL of MRS agar (Difco, Detroit, MI, USA). Once solidified, 10 µL of supernatant were pipetted on the agar surface. The plates were incubated at 30 °C for 18 to 24 hours and after this period it was possible to observe zones of inhibition.

3. Results and discussion

In the monitoring process of the cellular growth, the relationship between D.O. (600 nm) and dry mass (mL) was observed after filtration of the growth medium through a 0.22 µm membrane (Millipore).

The results shown at Figure 1 enable a comparison between the cellular growth curves of *Lc. lactis* CECT 4434, cultivated in MRS medium supplemented or not (control) with fructooligosacharides (MRS+FOS), polydextrose (MRS+PD) and inulin (MRS+IN).

Upon analyzing the growth curves regarding the cellular concentration, a behavior similar may be observed in terms of *Lc. lactis* CECT 4434 biomass formation for MRS (control), (MRS+FOS) and (MRS+PD) growth media, with values obtained at the tenth hour respectively of 0.94, 0.88 and 0.86 (g/L). On the other hand, under a prebiotic supplement, such as with (MRS+FOS) and (MRS+PD), there was an increase on the formation of biomass starting at 10 hours in contrast with MRS medium with no supplement.

It is evident that the cultivation of *Lc. lactis* CECT 4434 supplemented with inulin (MRS+IN) presented influence over the increase in the biomass production during the 48 hour cultivation. The cellular concentration values of *Lc. lactis* CECT 4434 with inulin as a supplement (MRS+IN) and with no supplement (control) were 1.48 and 0.98 (g/L) respectively.

These results highlight that the biomass production for *Lc. lactis* CECT 4434 is higher when supplemented with inulin, corresponding to the studies conducted by Oliveira *et al.* (2011), Oliveira *et al.* (2012) and Likotrafiti *et al.* (2014), which reported similar results regarding the capacity to increase the cellular count of probiotic microorganisms in cultivations supplemented with inulin.

In addition, in recent study, Kondepudi *et al*. (2012) demonstrated that prebiotic oligosaccharides, such as FOS and GOS may be used as a promising culture medium to optimize the production of probiotic *Bifidobacterium* species. We also observed an increase in the concentration of the *B*. *breve*, *B*. *lactis* and *B*. *longum* biomass, as well as of some BAL, for instance, the *Lactococcus lactis*. The latter showed antimicrobial activity against *Clostridium difficile*.

The effect of the FOS metabolism, extracted from corn silage and molasses, by *Lactobacillus* strains, in the production of bacteriocin, was investigated by Muñoz *et al*. (2012). In this study, it was confirmed that the different lactic acid bacteria LAB, for instance, *Enterococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* were able to use the FOS determined by the count of viable cells (CFU) successfully. An increase in viable cells count of BAL in the MRS growing medium, supplemented with FOS, was verified, unlike MRS culture medium without supplementation (control), which had a lower score.

Makelainen *et al*. (2010) evaluated the ability of different prebiotics, such as FOS and xylooligosaccharides (XOS), as potential candidates for carbon source to probiotic bacteria of the genres *Bifidobacterium* and *Lactobacillus*, as well as their spectrum of action to possible intestinal microbe pathogens, such as *Eubacterium*, *Bacteroides*, *Clostridium*, *Escherichia coli*, *Salmonella* and *Staphylococcus* in pure cultures. These authors could identify bifidobacteria that had preference for oligosaccharides, thus showing that the culture medium containing FOS and XOS, which have different degrees of polymerisation (DP) showed satisfactory fermentation, especially in strains of *B*. *lactis* and *B*. *adolescentis*, the *Lactobacillus*, in their turn, did not metabolize XOS efficiently. The same authors showed, in research published in 2010, that the FOS and GOS were fermented by a wide range of tested microorganisms, especially the *Bifidobacterium* and *Lactobacillus*, denoting the non-selectivity of FOS in pure culture studies. The researchers emphasized that the FOS are not selective only to the genera considered beneficial, but also to other intestinal micro-organisms, such as *Bacteroides spp*., *Clostridium spp*., and *Eubacterium spp*.

Mei *et al*. (2011) attributed the growth of probiotic bacteria to the influence of the FOS, which is directly related to the composition of FOS, like the β(2→1) fructans of short chains, once these greatly support the growth of probiotic microorganisms, when compared to the longer-chained β(2→1) fructans. Therefore, the authors could confirm that all β(2→1) fructans with glycosidics links are bifidogenic.

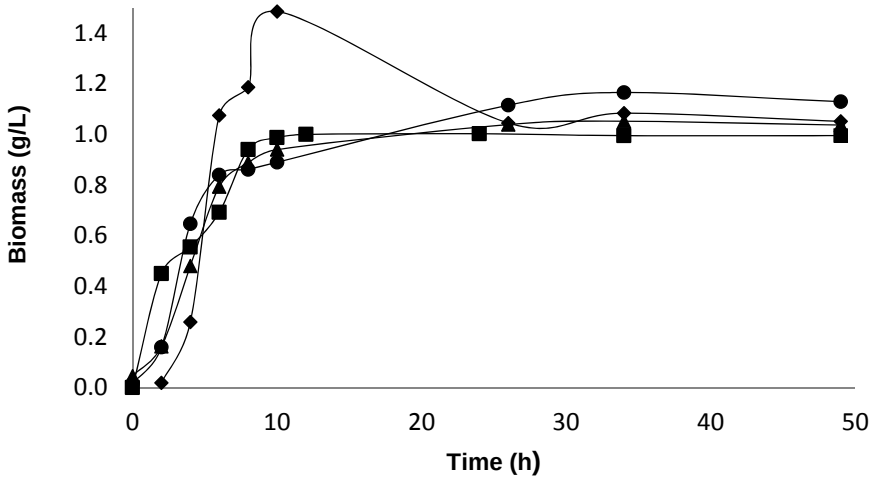

Figure 1. Comparison between the cellular growth curves for Lactococcus lactis subsp. lactis CECT 4434 in MRS medium (control: ■*) supplemented with (20g/L) of polydextrose (MRS+PD*: ●*), (20g/L) of fructooligosacharides (MRS+FOS*: ▲*) and (20g/L) inulin (MRS+IN*: ◆*).*

Upon observing the cellular concentration of *Lc. lactis* CECT 4434 cultivated in (MRS+IN) as a function of time, it is possible to observe a potential growth during the first 8 hours, reaching the peak biomass production at the eighth hour, with a dry mass value of 1.48 (g/L). Between 8 and 10 hours, the strain of *Lc. lactis* CECT 4434 presented a decrease in cellular production, reaching a dry mass value of 1.04 (g/L), and then presenting stability in cellular growth until 48 hours. These results are in accordance with a recent study conducted by Kassim *et al.* (2014) regarding the effect of chicory leaves, which contain inulin, as a growth medium for cultivating microorganisms from the *Lactococcus* and *Bifidobacterium* genera*.* The authors have identified a direct relation between the microorganisms' growth, especially those of the *Lactococcus* genus, in a growth medium containing inulin. Similar results have been reported by Oliveira *et al.* (2012), with inulin being among the best functional prebiotic ingredients for preparation of probiotic fermented milk, both for pure cultures and for binary co-cultures. In this study, the authors reported that the addition of inulin significantly reduced the time needed for fermentation, presenting higher biomass growth and an increase in the levels of diacetyl, acetone, lactic acid and acetic acid in both cultures (pure and/or co-culture).
Mei *et al*. (2011) have based themselves on different linear chain compositions of FOS and inulin so as to assess how those are used by different probiotic strains. Therefore, the authors used chicory and oat as a source for FOS and inulin, observing that the twelve probiotic strains tested, from the *Bifidobacterium, Lactobacillus* and *Pediococcus* genera, were capable of metabolizing FOS and inulin so as to optimize the biomass production of these strains.

3.1. Effect of the addition of different prebiotics in the cellular growth kinetics of *Lactococcus lactis subsp. lactis* CECT 4434.

The data for maximum specific speed for growth (μ_{max}) and the generation times (t_g) for *Lc. lactis* CECT 4434 in MRS medium supplemented or not with prebiotics.

Table 1. Values for maximum specific velocity for growth (μ_{max}) *for Lactococcus lactis subsp. lactis and generation time* (t_g) *using MRS medium in the presence of polydextrose, fructooligosacharides and inulin.*

Growth media*	Maximum specific speed for growth (μ_{max}) h^{-1}	t_g(h)
MRS	0.13±0.02[a]	2.66±0.08[d]
MRS + FOS	0.48±0.02[b]	1.42±0.04[c]
MRS + PD	0.94±0.03[c]	0.75±0.05[b]
MRS + IN	1.01±0.03[d]	0.68±0.06[c]

Values followed by the same letter in the same column are not different from each other, with probability of 5% by Tukey's test. *MRS (*Man, Rogosa and Sharpe*); PD (poydextrose); FOS (fructooligosacharides) and IN (inulin)

It is possible to observe that the maximum specific speed for growth (μ_{max}) in MRS medium and no supplement (control) was 0.13 h^{-1}, i.e. the lowest value among the tests, while the growth media supplemented with 20g/L of fructooligosacharides (MRS+FOS), polydextrose (MRS+PD) and inulin (MRS+IN) presented μ_{max} values of 0.48, 0.94 and 1.01 h^{-1}, respectively. These results showed that the addition of prebiotic ingredients to the MRS medium in combination with the probiotic strain *Lc*. *lactis* CECT 4434*,* positively influenced the metabolism, not only increasing the biomass concentration, but also favoring higher values for growth specific speed, especially in the case of inulin. A similar result was observed by Oliveira *et al.* (2012), Sims et al. (2014) and Saad et al. (2013), who studied the symbiotic interaction of inulin as an stimulating factor for the growth of probiotic microorganisms.

Upon analyzing the generation time (t_g) for *Lc*. *lactis* CECT 4434 in a MRS medium supplemented or not (control) with polydextrose (MRS+PD), fructoligosacharides (MRS+FOS) and inulin (MRS+IN), it is possible to highlight that t_g is inversely proportional to a μ_{max}, that is to say, the lower the growth specific speed, the higher is the generation time for the microorganism in question. The strain of *Lc*. *lactis* CECT 4434 cultivated in MRS (control) multiplied every 2.65 h. On the other hand, in a MRS medium supplemented with inulin (MRS+IN), the strain multiplied every 0.68 h. These results indicate that the addition of prebiotics, specially polydextrose and inulin, reduced the generation time for the probiotic strain in contrast to the control.

3.2. Bacteriocin antimicrobial activity

Upon testing the antimicrobial activity of the supernatant obtained by treating the growth medium for *Lc. lactis* CECT 4434 in comparison with *L. sakei* ATCC 15521, a formation of inhibition zones which varied as a function of time was observed. The production of bacteriocin begins within 6 hours of cultivation, generating halos of 12.55 mm in diameter (average value obtained in MRS medium). This phase of the cultivation includes the exponential phase for the strain *Lc*. *lactis* CECT 4434. After 10 hours, the inhibition zones diameter increase to 15.55 mm, but decrease to 14.85 mm at 16 hours, probably due to a peptidic instability caused by the organic acids produced by the strain in question. The values obtained with the addition of prebiotics to MRS medium were on average 12% lower compared to the control (without supplementation). Gomes *et al*. (2012) observed a reduction of bacteriocin activity by approximately 50% and 62% when the inulin and oligofructose were added to MRS medium. On the other hand, Chen et al. (2007) reported that the addition of FOS and trehalose increased the bacteriocin production by *Lactococcus lactis* ssp. *lactis* C101910.

4. Conclusion

The different prebiotic ingredients used as a growth medium for *Lc*. *lactis* CECT 4434 presented a positive influence over the growth of microbial biomass. With the fermentation process, it was possible to observe that the prebiotic ingredients foster cellular growth of *Lc*. *lactis* CECT 4434.

On the different tests conducted, it is possible to observe that the prebiotic ingredients, especially inulin, provided an increase in the maximum specific speed for growth (μ_{max}) and generation time (t_g).

Furthermore, the antimicrobial activity of *Lc*. *Lactis* CECT 4434 in supplemented cultivations with prebiotic fibers, in contrast with the strain *L. sakei* ATCC 15521, shows promise as food products may be created based on this symbiosis, which may be of commercial interest due to not having chemical preservatives, with the bacteriocin playing that role.

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