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THE IMPACT OF POTENTIAL PREBIOTICS INULIN, OLIGOFRUCTOSE AND POTATO STARCH ON THE GROWTH OF *Lactobacillus casei*

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Abstract

Prebiotics are increasingly being added to probiotic foods to enhance the survival and growth of probiotics. This study examines the ability of the Lactobacillus casei strain to use various prebiotics (potato starch, chicory inulin and oligofructose) as carbon sources for cell growth. Therefore, four culture media were prepared for the growth of specific lactobacilli. Three of these media were modified. The original medium recipe De Man Rogosa and Sharpe (MRS) was respected, replacing only the carbon source - glucose, resulting a starch MRS broth, MRS broth with inulin and MRS broth with oligofructose. As standard it was used glucose from the MRS broth. The growth of biomass and the increase in the amount of lactic acid were analyzed. Spectrophotometric measurements of optical density were made for biomass growth, and for the determination of lactic acid growth, the acidity of the media was analyzed by monitoring the pH and the increase of the titratable acidity. After standardization of the probiotic culture at 37°C for 24 hours and inoculation in modified MRS media, the growth of the Lactobacillus casei strain was monitored for 12 hours once every 2 hours. It has been observed that glucose replacement with the three medium prebiotics favors bacteria growth during incubation at 37°C and resulted in an improvement in L. casei strain culture.

Key words*: probiotics, prebiotics, Lactobacillus casei, inulin, starch, oligofructose.*

INTRODUCTION

According to FAO/WHO (2001) the probiotics are known as living microorganisms which confers an improvement to the host’s health when they are administered in adequate quantities. Probiotics can aid the human organism by maintaining healthy bowel microbes, stimulating the immune system, inhibiting the production of pathogenic microorganisms, ameliorating constipation, improving calcium absorption, antimicrobials synthesis vitamin synthesis, etc. However, to receive all the beneficial effects, they need to survive the journey through the upper gastrointestinal tract to the intestine (Ashwar et al., 2018). The main obstacles to the survival of ingested probiotics are the stomach’s high pH and biliary salts secreted in the duodenum (Ashwar et al., 2018). Therefore, it is necessary to ensure the safeness of probiotics before and after consuming them to assure the user of their beneficial effects, using encapsulation techniques for incorporation into different foods. However, in some cases in order to improve the protection and viability of probiotics it is required the incorporation of natural prebiotics, because, encapsulation does not secure the overall viability of microorganisms (Peredo et al., 2016).

Prebiotics may be described as "non-degradable food ingredients which, when consumed in sufficient quantities, selectively stimulates the growth and/or activity of one or a limited number of micro-organisms in the intestine resulting in documented health benefits"(Burgain et al., 2011). Prebiotics improve the survival in the gastrointestinal tract of probiotics, their viability and vitality, their attachment and subsequent growth in the intestine (Burgain et al., 2011), The daily consumption of fruits, grains and vegetables can be a natural approach to obtain prebiotics (Kerry et al., 2018; Quigley, 2018).

Starch is widely used in the food industry and other industries and also, it is a major component of our diet. Starch is a polysaccharide composed of a large number of glucose units linked by glucosidic bonds. Amylopectin and amylose is the composition of starch. Amylopectin is composed of linear chains α-(1- →4) with linked chains α- (1→ 6), (Gänzle

and Follador, 2012). Amylose is a linear glucose chain α- (1→4) with a plant-specific degree of polymerization of 200-6000. To have the desired properties for various applications including encapsulation and controlled release of the capsule contents, the natural starch is commonly modified by physical, chemical and/or enzymatic methods (Zhu, 2017; Khorasani and Shojaosadati, 2017).

Inulin is a natural polysaccharide, a natural fructan (Figure 1) [α-D-glucopyranosyl-(β-D-fructofuranosyl)] (n-1) –D-fructofurano side (Ni et al., 2019), considered a food fiber which, due to high resistance to gastric acid and pancreatic enzymes is frequently used as a food ingredient. In addition, there are known some studies (Cooper and Steele, 1988, 1991; Cooper, McComband Steele, 1991; Silva, Cooperand Petrovsky, 2004) that shows that it can be used as a functional and prebiotic ingredient and it also has remarkable biological effects with immune properties. Even if inulin is extensively fermented by bacteria in the large intestine it cannot be absorbed into the small intestine and is not hydrolyzed due to its chemical structure. Some papers (Castelli et al., 2008) have found an antigenotoxic and anticototoxic effect in addition to its prebiotic nature. With good organoleptic properties, inulin is recognized as an attractive constituent for structuring low-fat foods (Ni et al., 2019; López-Castejón et al., 2019; Karimi et al., 2015). Inulin is usually comercially extracted from chicory, dandelion, agave, dahlia etc. Crops such as banana, wheat and onion is also a source of inulin even if it is found in lower amounts (Ni et al., 2019; Drabińska et al., 2016).

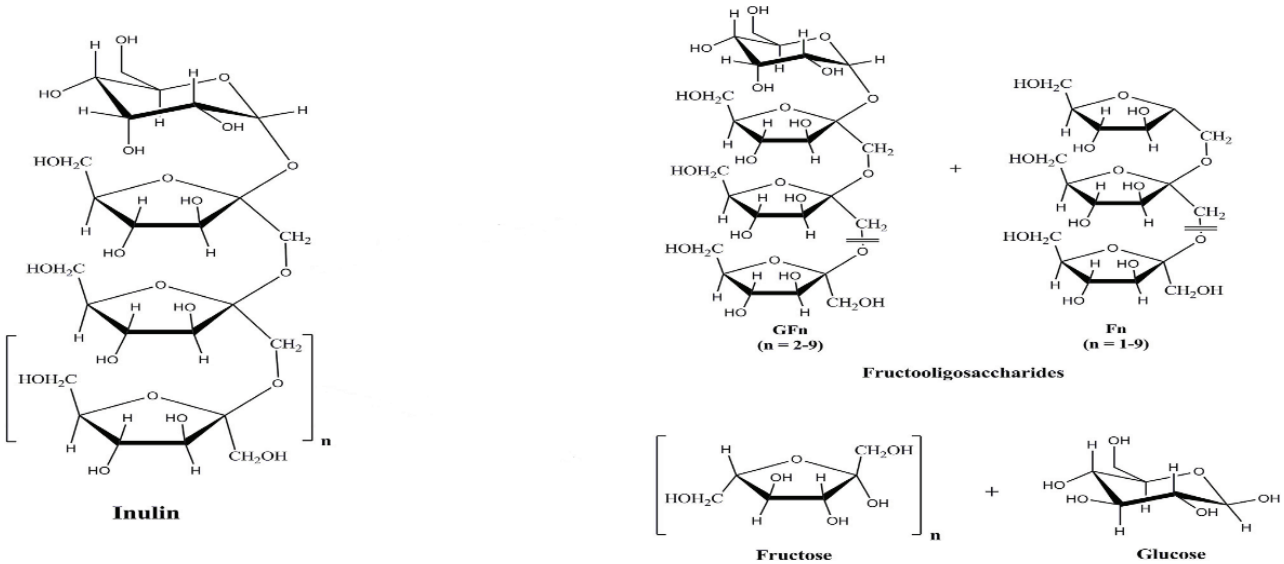

Figure 1. The chemical structures of carbon sources: inulin, oligofructose, glucose and fructose (Singh et al., 2017)

Oligofructosesor fructo-oligosaccharides (FOS), presented in Figure 1, are a reserve polysaccharide that offers an interesting combination of nutritional and technological properties for the food industry. They selectively stimulate the growth of lactobacilli and bifidobacteria, which improves human health. These compounds are considered a functional food ingredient that can be used as food and prebiotic fiber. This type of prebiotics regularly consumed has positive effects on human health. FOS can reduce cardiometabolic risk by lowering triglycerides and cholesterol levels, modulating hyperglycemia, preventing colorectal cancer (Ambalam et al., 2016), increasing intestinal absorption of minerals (Lobo et al., 2011) and improving immune system efficiency (Peshev and Van den Ende, 2014; Lopes et al., 2016).

Considering the necessity of prebiotics encapsulation in capsule in order to ensure the survival of probiotics in their direction through the digestive tract, as well as the fact that the aforementioned prebiotics can be used as a carbon source for these microorganisms, we consider it useful to test the compatibility of these prebiotics with the growth of some probiotic strains. Therefore, the objective of this study was to analyse the capacity of the *Lactobacillus casei* strain to use various

prebiotics (potato starch, chicory inulin and FOS) as carbon sources for cell growth/ multiplication.

MATERIALS AND METHODS

Materials

The prebiotics used were soluble potato starch (Sigma-Aldrich, Germania), oligofructose (FOS) and chicory inulin (Sigma-Aldrich, Germania).

The culture medium

Four types of culture media were prepared. The first culture medium, used as a standard, was De Man-Rogosa-Sharpebroth (MRS broth) (De Man et al., 1960). The other three media were MRS modified by replacing glucose from the original recipe as carbon source with soluble potato starch, chicory inulin and FOS, in the same ratio (20 g/L). Using a vortex homogenizer (Vortexer, Heathrow Scientific®LLC) the media was homogenized for 15 minutes at 1400 rpm. The pH of the media was adjusted before sterilization to 6.2 using NaOH and HCl. The media were sterilized for 15 minutes at 121°C.

Preparation of inoculum and microorganisms used

The *Lactobacillus casei* 431 strain used in this study was purchased from Christian Hansen. *L. casei* 431 is a registered trademark of Chr. Hansen. Prior to use, *L. casei* was incubated at 37°C for 24 hours and activated to standardize in MRS broth. All solutions and media were prepared with distilled water (Aquatron A4000D, Cole-Parmer Ltd).

Determining the beginning of the logarithmic phase

From the 24 h of *Lactobacillus casei*, inoculations were made in triplicate in the 4 types of MRS broth and incubated for 12 h at 37°C. Absorbance was determined at 0 (after inoculation) and at 2 hours intervals by spectrophotometry at 600 nm (JENWAY 6400 spectrometer). The bacterial growth curve is plotted in Figure 2, so the beginning of the logarithmic phase was determined. Three test tubes from each medium were prepared for each reading range.

Consumption of prebiotics - methods of analysis

Measuring biomass growth (cellular)

Optical density at 600 nm was used as a parameter related to the amount of biomass (Peredo et al., 2018). For a period of 12 hours, at every 2 hours optical density was monitored using a visible UV spectrophotometer (Jenway 6400, Jenway) (Peredo et al., 2018). Figure 2 shows the calibration curve of the McFarland standard whose regression coefficient is R^2=0.978.

Determination of pH and acidity of the medium

Within 12 h the determinations of the two parameters were made once every 2 hours. The determination of pH was done immediately afterdetermining the optical density using the pH-meter (Hach Lange HQ11D Digital pH meter).

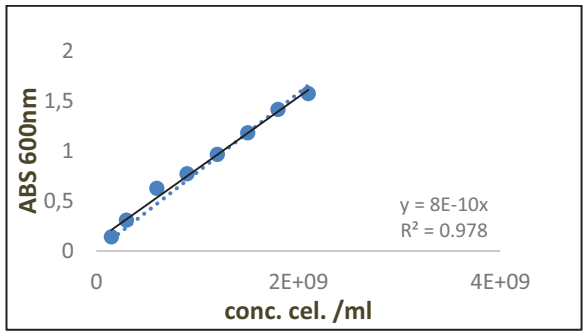

Figure 2. The calibration curve of the McFarland standard

The medium acidity was determined by titration with an alkaline solution of NaOH 0.1 N until the sample was neutralized in the presence of phenolphthalein as an indicator. In the medium there can be observed a gradual decrease of pH due to microbial fermentation of the carbon source (glucose, FOS, inulin and starch). The carbon source is transformed in lactic acid which is responsible for the medium acidity.

Statistical analysis

For the statistical evaluation of the data, the ANOVA test followed by the post-hoc Turkey test was used. It was considered as a statistically significant difference at a value of $p<0.05$.

Statistical replication was provided by using 3 sets of probiotic culture tubes for each type of

culture medium, inoculated and analyzed independently.

RESULTS AND DISCUSSIONS

The prebiotics effect on the cellular concentration increase

For metabolic energy, lactobacilli use homofermentative or heterofermentative fermentation of carbohydrate because they have complex nutritional requirements for fermentable carbohydrates, nucleic acids, amino acids and other substrates.

The growth of the *Lactobacillus casei* strain in the 4 types of culture media is shown in Figure 3.

Gänzle and Follador (2012) state that many of the lactobacilli species metabolize a wide variety of different carbon sources, as well as all major oligosaccharide classes, but the most representative are *Lactobacillus plantarum, L. acidophilus* and *L. casei.*

They say that depending on the capacity of individual strains and lactobacilli species the metabolism of oligosaccharides differs substantially (Gänzle and Follador, 2012).

Figure 3-D show that replacing in the medium the glucose with potato starch favors bacterial growth during incubation at 37°C. The bacteria increased more ($p<0.05$) compared to the addition of inulin, oligofructose and glucose in the presence of potato starch. The same observation is also made by Peredo et al. (Peredo et al., 2018) where it examines the consumption of prebiotics (starch, psyllium and chicory inulin) by *L. plantarum* Lp17*, L. plantarum* Lp33 *and Lactobacillus casei* Shirota (Peredo et al., 2018).

Inulin, the most abundant carbohydrate after starch [18], also favored the growth of bacterial strains Figure 3-B statistically significant comparing with the glucose medium. The same can be noticed in the case of oligofructosis Figure 3-C.

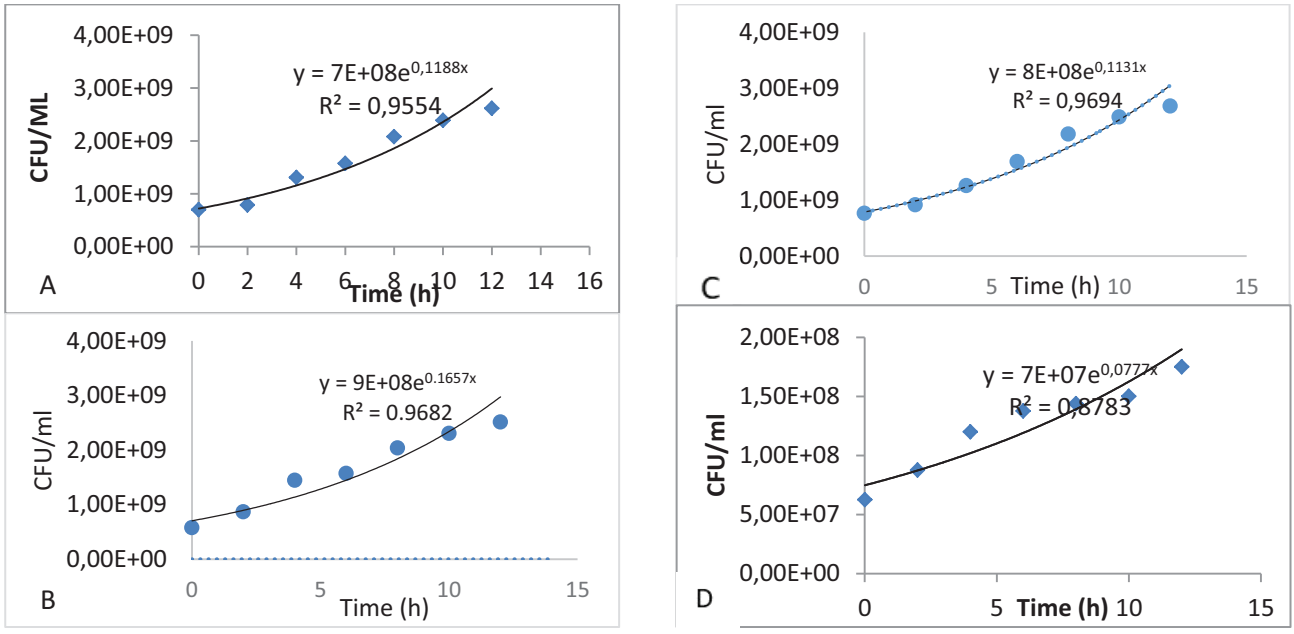

Figure 3. The growth of the *Lactobacillus casei* strain in the 4 types of culture medium: A-glucose, B - chicory inulin, C- oligofructose, D - potato starch

Comparing the growth of biomassin the mediums where glucose was replaced with oligofructose and inulin (Figure 3-B,C), there was no statistically significant increase between them, but there was a significant increase from the medium that had glucose (Figure 3-A), as carbon source. Polysaccharides of fructan type, FOS and inulin consisting of β-d-fructofuranosyl (β→d-Fruf) bound (2→1) with a glucose residue bound by α→2) bonds.

It can be used GFn as the general structure of fructans, where 'n' represents the number of fructosyl, F represents fructose and G represents glucose (Singh et al., 2017; Shoaib et al., 2016). Fructans differ in degree of polymerization (DP) or chain length, therefor inulin has DP ranging from 10 to 65 units of

fructose, but the most common are molecules with DP values between 12 and 15 and FOS are smaller molecules with a DP<9 and (Singh et al., 2017).

Inulinase, hydrolysis enzymes, produced by probiotic strains specifically act on β-2,1 links of inulin to produce fructose or FOS (Ni et al., 2019; Singh et al., 2017). Kuzuwa et al. (2012) showed that a strain of *L. casei* IAM1045 grows in the medium containing FOS or inulin, produces extracellular in the culture medium, depending on fructan degrading inulin proteins. Su et al.(2007) reported that in a basal medium supplemente with inulin but also with FOS, are able to grow *Bifidobacterium lactis* and *L. casei*, likewise, Huebner et al. (2008) ran a study to choose which prebiotics (including inulin and FOS) supported selective growth of bifidobacteria and lactobacilli.The study results indicates the fact that only specific combinations of prebiotics and probiotics yielded good results; which includes *L. paracasei* 1195 cultivated on inulin. Kanjan and Hongpattarakere (2017) reported that inulin couldn't be degraded by the most well-known probiotic lactobacilli species like *L. rhamnosus* GG*, L. delbrueckii, L. plantarum and L. casei* Shirota. However, this particular substrate of prebiotic could be digested only by a few strains of *L. paracasei* and *L. casei* (Takagi et al., 2014; Kanjan and Hongpattarakere, 2017), so Karim's assertion that only certain combinations of probiotics and prebiotics can produce good results applies.

Lopes et al. (2017) have conducted studies on inulin and isolated FOS from *Stevia rebaudiana* Bertoni using it as an energy source for bifidobacteria and lactobacilli and found that for inulin there was no statistically significant ($p<0.05$) increase in biomass compared to control medium as it has a degree of polymerization = 12 (Singh et al., 2017). In contrast, for FOS, it was found that each strain used FOS of *S. rebaudiana* with varying intensity. This is in line with the overall knowledge of substrate specificity and strain (Moreno-Vilet et al., 2014). Isolated from the same source, FOS molecules with DP<6 proved to be a better use as a substrate for probiotic strains than inulin molecules (DP = 12). As for the titratable acidity analysis, the evaluation of this parameter supports the cell growth values determined by spectrophotometric measurements.

In contrast, microorganisms grown in starch as carbon sources (Figure 3-D) exhibited less growth compared to glucose. Starch has a high molecular mass and a high degree of polymerisation. Generally, starch is degraded by amylases, which are not common in lactic bacteria. But a study by Khorasani and Shojaosadati (2017) shows that starch can be used to protect probiotics against digestive enzymes such as pepsin, proteases and lipases because they can not hydrolyze it. Furthermore, probiotics, entrapped in a starch coated microcapsule, can hydrolyse the starch coating layer when they reach the large intestine. Microbial hydrolysis starts from within the microcapsule to the outside, where the coating is located. Thus, enzymatic and microbial hydrolysis of starch leads to the release of encapsulated probiotics in the intestine. (Khorasani and Shojaosadati, 2017).

With the increase in cellular concentration, the pH is decreased at each reading interval (Table 1). Thus, after 12 h of incubation, the pH from 6.2 decreased to 4.45 for inulin, to 4.46 for FOS, and 5.97 for starch as a result of consumption of the carbon source and, probable, its transformation into lactic acid (Pimentel-González et al., 2009).

Table 1. The pH variation during 12 h of monitoring

Media type	Time (h)													
	0		2		4		6		8		10		12	
	Avg*	SD	Avg*	SD	Avg*	SD	Avg*	SD	Avg*	SD	Avg*	SD	Avg*	SD
Starch	6.033	0.012	5.837	0.032	5.920	0.078	5.817	0.006	5.943	0.142	5.950	0.111	5.970	0.070
FOS	5.603	0.006	5.457	0.012	5.240	0.035	4.973	0.023	4.737	0.006	4.600	0.017	4.457	0.021
Inulin	5.527	0.006	5.330	0.017	5.163	0.023	4.980	0.010	4.763	0.021	4.603	0.023	4.450	0.010
Glucose	5.497	0.006	5.367	0.006	5.193	0.006	4.967	0.015	4.747	0.012	4.553	0.006	4.417	0.015

*avg - average

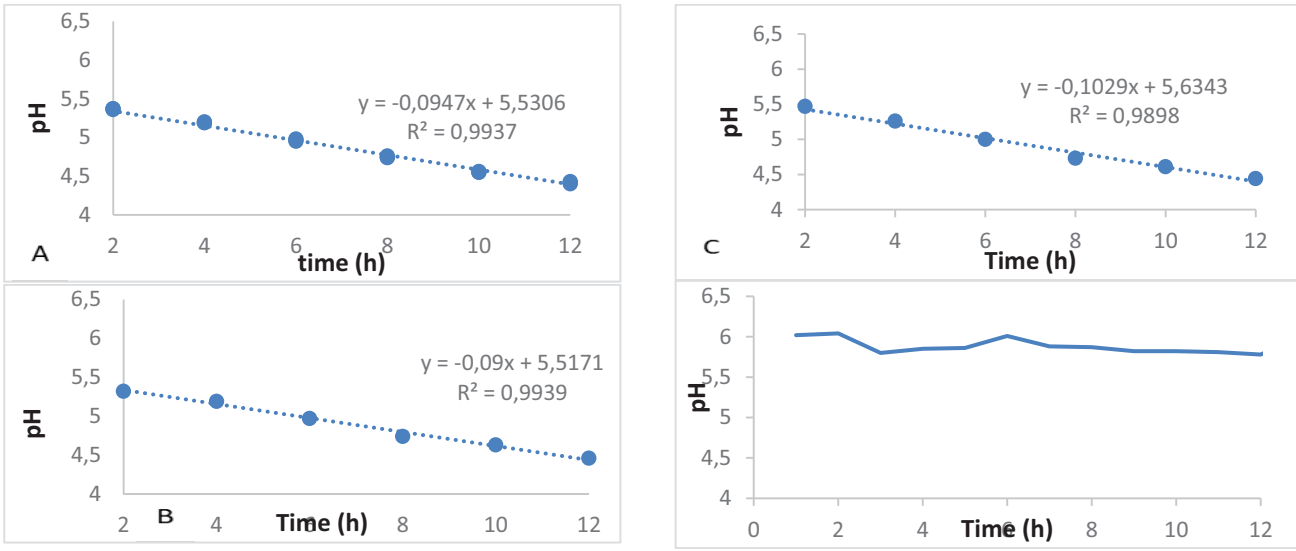

Figure 4. The evolution of pH as a consequence of the growth of *L. casei* on different culture media: A - glucose, B - chicory inulin, C - oligofructose, D - potato starch

Mathematical modeling of data

Experimental data were modeled using exponential equations. It has been noticed that regardless of the carbon source used, whether we are talking about inulin, FOS or starch, the growth of biomass was exponential.

Regarding the acidity of the medium we can observe a linear increase with the production of lactic acid Figure 4, except for culture medium supplemented with starch

CONCLUSIONS

In this study, three sources of carbon were evaluated in order to study the production of *L. casei*. The results showed that biomass production and substrate consumption increased significantly over the 12 h for culture medium supplemented with inulin and FOS. The three tested carbon sources have led to an improvement in strain cultivation expressed as CFU/ml. The results obtained lead to the possibility of using the tested prebiotics both as a nutritive substrate and as a probiotic encapsulation material.

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