Journal homepage: www.fia.usv.ro/fiajournal
Journal of Faculty of Food Engineering,
Ştefan cel Mare University of Suceava, Romania
Volume XVI, Issue 2 – 2017, pag. 67 - 74

THE PREBIOTIC POTENTIAL OF SOME CARBOHYDRATE SUBSTRATES ON THE GROWTH OF *Lactobacillus plantarum* AND *Lactobacillus rhamnosus*

Liliana LUCA[1], *Mircea OROIAN[1], Andrei LOBIUC[1]

[1]*Faculty of Food Engineering, Stefan cel Mare University of Suceava, Romania*
m.oroian@fia.usv.ro
*Corresponding author
Received 15th February 2019, accepted 29th June 2019

Abstract: Probiotic intake in human nutrition is particularly important for maintaining an optimal physiological state A de Man-rogosa-Sharpe (MRS) broth medium free of fermentable carbon source and a basic MRS broth was used to investigate the ability of the *Lactobacillus plantarum* and *Lactobacillus rhamnosus* strains to grow on selected prebiotic. Criteria for the evaluation of the use of prebiotics were the growth of Lactobacillus and acidification rate as compared against the results obtained in the control media. *Lactobacillus plantarum* and *Lactobacillus rhamnosus* were monitored for 10 hours in culture media with starch, inulin, glucose and media without glucose to assess the opportunity of sugar substrates as a support for the development of probiotics for micro-encapsulation. The largest number of final CFU was obtained for inulin, followed by oligo-fructose and starch. The oligo-fructose and inulin did not reach the stationary phase after 10 hours, while the stationary phase for starch, glucose and medium without glucose was recorded at 8 hours, 6 hours and 6 hours, respectively. The development of lactic microorganisms was also observed by decreasing the pH in the culture medium during monitoring.

Keywords: probiotics, prebiotics, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*

Introduction

In the last years, the interest in better living and improved health led to a significant increase in demand for new health-promoting foods, including probiotic functional foods [1]. The word "probiotic" comes from the Greek words "pro" and "bios" which together means "for life" and are therefore widely considered to be beneficial microorganisms. According to the World Health Organization, probiotics must be administered in adequate amounts (10^7 CFU g^{-1} or CFU/ml) to confer health benefits to the host (FAO/ WHO, 2001) at the time of consumption [1], [2]. In order to be able to offer these benefits, microorganisms must survive during harsh conditions throughout the food production cycle (such as pH, temperature, fat and humidity), storage as well as stress in the gastrointestinal tract [3]. Therefore, new approaches which could protect and deliver probiotics are required, for instance, microencapsulation [4] (Wu, Y., & Zhang, G. 2018). Research trends are now increasing with regard to the combined use of prebiotics and microencapsulation to enhance and maintain the viability of probiotics 1 - Ningtyas et al., 2019. Some authors such as [5], [6], emphasize the fact that many of food oligosaccharides and polysaccharides have been claimed to have prebiotic activity, but not all dietetic carbohydrates are prebiotic. The majority of probiotic candidates are non-digestible oligosaccharides such as lactulose, refinose, fructooligosaccharides, lactose, saccharides, isomaltooligosaccharides, etc., but also include highly polymerized saccharides such as inulin, resistant starch, pectin, etc. It was also shown that this combination of probiotics and prebiotics, known as symbiotic, improves probiotic proliferation in the intestine and helps to modify the intestinal community [5], [6].

Probiotics. Regarding the known probiotic microorganisms, [7], known species of lactic acid bacteria (LAB) (e.g., *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Enterococcus*), *Bifidobacterium*, *Bacillus*, and yeast, are considered to play important roles in maintaining the intestinal ecosystem and stimulating their host's immune system [8]. According to *Figure 1*, *Lactobacillus*, under the LAB group, was the earliest discovered probiotic. In their paper, [9] - König and Fröhlich, 2009 stated that this genus of *Lactobacillus*, Gram-positive bacteria, comprises 183 species and they are used in many industrial processes such as the production of preservatives, the production of food flavors, pharmaceutical industry, the cosmetics industry, etc. Regarding the selection of probiotic microorganisms, it usually follows a strategy similar to that shown in *Figure 2* [7].

Prebiotics. In 2016, a panel of experts summoned by the International Scientific Association for Probiotics and Prebiotics (ISAPP) defined prebiotics as "a substrate selectively used by microorganisms that provide health benefits" [10]. Prebiotics such as inulin, oligofructose, starch, lactulose etc. are indigestible carbohydrates (not digested by human enzymes but act as a fermentable substrate for probiotics in the colon) [1] that selectively promote the growth of probiotics and demonstrate that prebiotics have the potential to alter in a positive manner the composition of intestinal microbiotherapy [4].

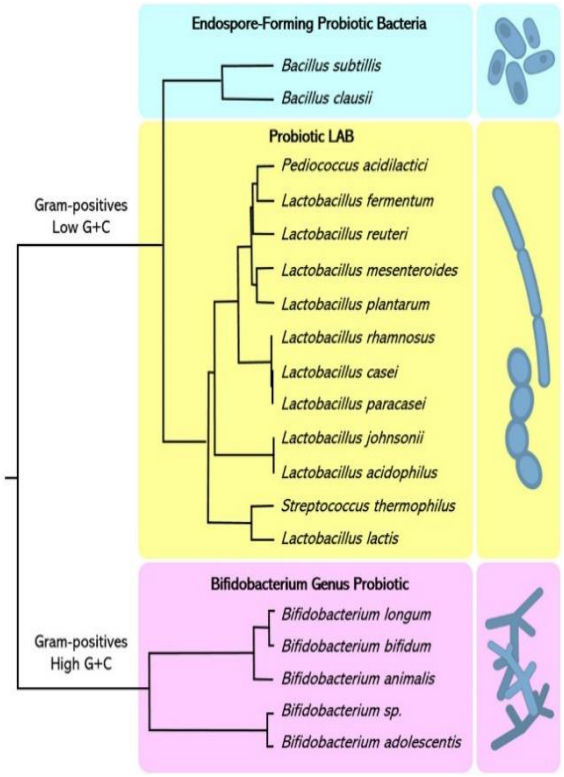

Fig.1. the phylogenetic relationship of the different probiotic bacterial groups through16S rRNA gene sequences retrieved from GenBank database. [7]- Pereira et.al 2018

Studies conducted on the growth of some *Lactobacillus* strains (*L. paracasei* KTN-5, *L. delbruckii* TU-1, *L. plantarum* 22A-3), inulin and fructose showed that the strain of *L. plantarum* 22A- 3 could use fructose but not inulin. Furthermore, in co-culture with the other 2 strains from the study grown on inulin, the growth of the *L. delbrurckii* strain was not as affected as the *L. paracasei* strain [5].

[11] - Lopes et al., conducted a study in 2017 on the prebiotic activity of inulin and oligofructose extracted from *Stevia rebaudiana* on seven strains of bifidobacteria and lactobacilli.

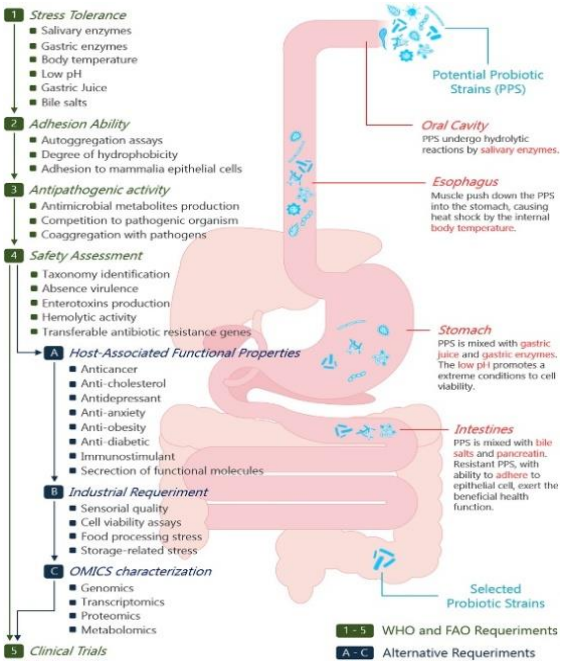

Fig. 2. Screening approaches used to characterize probiotic strains according to WHO / FAO and studies quoted by [7] - Pereira et al. 2018

They found that in vitro, oligofructose molecules with a low degree of polymerization are preferred by microorganisms in comparison with inulin that has a higher degree of polymerization.
This study investigated the influence of prebiotics used as a carbon source on the increase in the biomass *of Lactobacillus rhamnosus* and *Lactobacillus plantarum*.

2. MATERIALS AND METHODS

Media and substrates

The prebiotics used were: inulin in chichory (INU), potato starch soluble (STH) and oligofructose (OLI). The Man-rogosa-Sharpe (MRS) agar (Sigma Aldrich, Germany), MRS broth from Sigma-Aldrich (Saint Louis, USA) was used for cultivation in 4 variants. For control variant, basic MRS recipe, including glucose (20 g/l), was followed, while, for testing carbohydrate substrates, the glucose in basic MRS recipe was replaced by different amounts of the respective substrates (20 g/l of each). Chicory inulin, potato starch and oligo-fructose have been used as a carbon source by replacing glucose in the original MRS recipe. As a negative control, a carbohydrate-free medium and an un-inoculated medium were used. For comparison of bacterial growth, the simple MRS broth was used. All the chemicals were purchased from Sigma-Aldrich (Saint Louis, USA).
Before being used, the modified culture media were adjusted at the pH of 6.2 and the MRS agar medium at 5.7, and then sterilized for 20 minutes at 121^{o}C.

Microorganisms and cultivations

The bacterial strains used in this study were *Lactobacillus plantarum* and *Lactobacillus rhamnosus (Bioprox RP 80, France, Lactobacillus cells count* = 7±2x10e10)
The lyophilized bacterial strains were cultivated in the MRS broth at 37^{o}C for 24h and then they were inoculated on the 4 modified media (medium MRS broth without carbohydrate source: MRS WG, MRS – INU, MRS – STH, MRS – OLI) and on a MRS broth. The last culture medium was used as a standard medium. The growth of the bacteria was quantified through plate counting. All assays were performed in triplicate yielding a total of 109 experiments.

Bacterial strains and preparation of bacterial suspension

The bacterial strains used in this study were *Lactobacillus plantarum* and *Lactobacillus rhamnosus,* these strains were obtained from lyophilised culture Bioprox RP80 (Noyant, France) distributed by SC Enzimes & Derivates SA (Romania). Lactobacillus cells count = 7 ± 2x10e10 CFU /dose. Bacterial strains were reactivated in MRS broth medium at 37 ° C for 24 h. From the reactivated culture (24 h) bacterial suspensions (1 ml) were used for inoculation in each test medium. Later, the media were incubated at 37 ° C for 12 hours and tested every 2 hours. All determinations were made in triplicates.

Prebiotic activity testing

Prebiotic activity, reported in this study, reflects the ability of some substrates (INU, STH, OLI) to support the growth of probiotic organisms using as a standard non-prebiotic substrate – glucose (MRS) and a carbon-free medium (MRS WG). Assuming that these carbohydrates (INU; STH; OLI) will be metabolized by probiotic strains such as glucose. Criteria for the evaluation of the use of prebiotics were also the acidification rate as compared against the results obtained in the control medium. Rate of acidification by these strains was determined as changes in pH.

Measurement of biomass growth

Optical density of culture media was measured (Jenway 6400, Jenway Ltd UK) after inoculation (zero time) every 2 hours, for 12 h. Bacterial growth was evaluated as the change in absorbance (at 600 nm) of the medium during 12 hours of incubation at 37 ° C, interpolated in a MacFarland standard curve, (Figure 3 with the regression coefficient of R^2=0,978. The change of *the acidity of the medium* was determined once every 2 hours for 12 h by measuring the pH using a pH meter (Hach Lange HQ11D Digital pH meter).

Viable cell counts

The growth of bacteria was quantified through plate counting. MRS agar medium was used as a control for bacterial growth and bacterial viability. MRS agar was inoculated from subculture starting at point 0 then once at 2 hours for 12h using serial dilution method. MRS agar plates were incubated at 37°C for 48 hours. All the inoculations were made in triplicates. After 48h colonies on plaques were counted and expressed as colony forming units per ml.

Statistical analysis

For the statistical evaluation of data, the ANOVA test followed by the post-hoc Tukey test was used ($p < 0.05$). Statistical replication was provided by using 3 sets of probiotic culture tubes for each type of culture medium, inoculated and analyzed independently and the same for MRS agar plates.

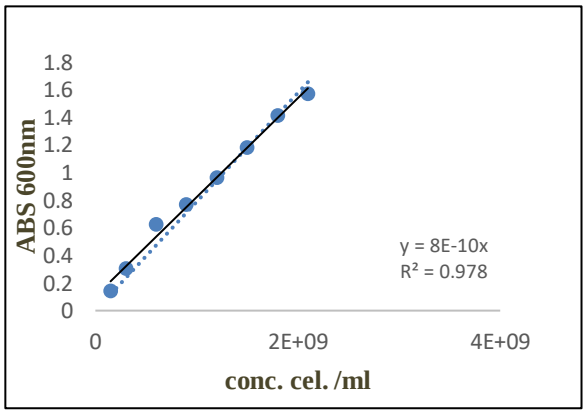

Fig. 3. The calibration curve of the McFarland standard

3. RESULTS AND DISCUSSION

Biomass growth

The number of bacterial cells after 0, 2, 4, 6, 8, 10 h of incubation is given in figure 4 and figure 5. In the first 4 hours of incubation, the growth of probiotic cells was observed in all 5 culture media.

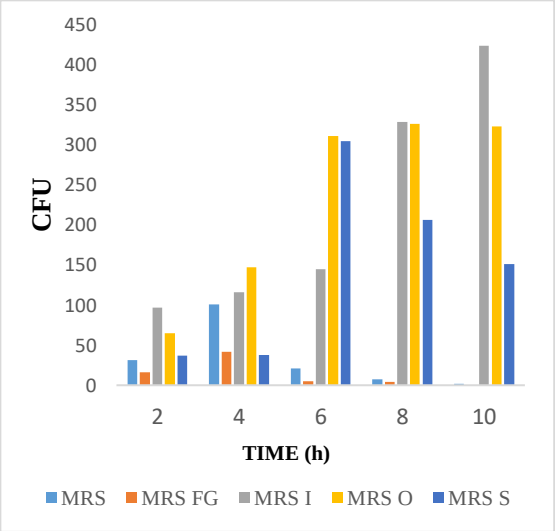

Fig. 4. Biomass growth

In comparison with the control cultures, in the case of MRS INU and MRS OLI followed by MRS STH cultures it was observed that *Lactobacilli* had a significant increase ($p > 0.05$).

After 6 h the culture of *L. rhamnosus* and *L. plantarum* reached the stationary phase on the MRS STH medium and on the culture media with INU and OLI it was still growing. On the other hand, it was observed that on culture media used as control media (standard), probiotics entered in a regression phase.

Growth rate

To analyze the growth rate of culture, the following formula was used:

$R_{x-y} = (V_y - V_x) / (T_y - T_x) \Rightarrow R_{x-y} = (V_y - V_x) / 2$ CFU / h,

where R = growth rate, V = 2 hours, T = reading interval = 2h, x and y time interval where y = x+2h .

Analyzing the growth rate of the probiotic culture (*Figure 6*) on each substrate, there was a sharp increase in MRS STH at the 3rd measurement (after 6 h of incubation). The culture entered the logarithmic phase with a growth rate of 133% as compared to the second measurement. By accumulating in this phase a maximum amount of biomass, the substrate was quickly exhausted and the culture went into the stationary stage of growth

Starch is composed of amylose and amylopectin. Amylose is a linear chain of glucose α- (1 → 4) with a plant-specific degree of polymerization of 200-6000. Amylopectin consists of linear chains α- (1 → 4) with linked side chains α- (1 → 6). Amylolytic degradation of amyloses by α- and β-amylase and amyloglucosidase leads to linked maltodextrins respectively, maltodegas and glucose. Hydrolysis of amylopectin requires amylopululanase or pullulanase to cleave the α-(1→6) branch points; the hydrolysis of amylopectin produces in addition α D-Glu-α- (1-6) -D-Glu (isomaltose) and oligosaccharides with bonds α-(1-→4) and α-(1-→6) mixed [12]-Gänzle and Follador (2012). According to a study conducted by [13]-Humblot et al. 2014, which cultivated *L. plantarum A6* on MRS STH, simple carbohydrate consumption takes place over the first 3 hours, followed by maltodextrins with 3 to 7 glucose units.

On the other hand, inulin and its partially hydrolyzed derivative oligofructose are made up of linear β-(2→1) glycosidic bonds of D-fructose (linkages that differ in a high (10–60) (inulin) and low (3–7)

(oligofructose) number of fructose monomers), often with a terminal glucose moiety that is linked by an α-(1→2) glycosidic bond, as in sucrose. Oligofructose can contain both chains of fructose (Fm type) and fructose chains with a terminal glucose unit (GFn type) [14, 15].

In a 2005 study, it was shown that the consumption of free fructose occurs within 4 hours of fermentation, oligofructose was preferentially metabolized during the exponential growth phase, and rapid degradation of long-chain inulin resulted in an increased concentration of fructose and sucrose after 8 hours of growth [16].

As for INU and OLI, the other substrates analyzed in this study, there were significant growth rates (p> 0.5) as compared to the second measurement (6h). Because probiotic culture had a growth rate of 82% on MRS OLI versus second reading and a growth rate of 7.5% versus the third reading, probiotic culture started the logarithmic growth phase at 6 h and entered the 10h stationary growth phase.

Regarding the growth rate on MRS INU, the logarithmic growth phase began at 4h but without the initiation of stationary growth phase. Some lactobacilli species can use inulin in two steps, first to degrade it to fructose and then to use the fructose molecules inside the cell [5].

Regarding the evolution of pH, after 10h of incubation, the results showed that with the increase in cellular concentration, the pH decreases at each interval reading (*Table 1*). Thus the pH from 6.2 decreased to 4.32 for inulin, to 4.27 for OLI, and 4.36 for STH as a result of consumption of the carbon source and, probable, its transformation into lactic acid [17].

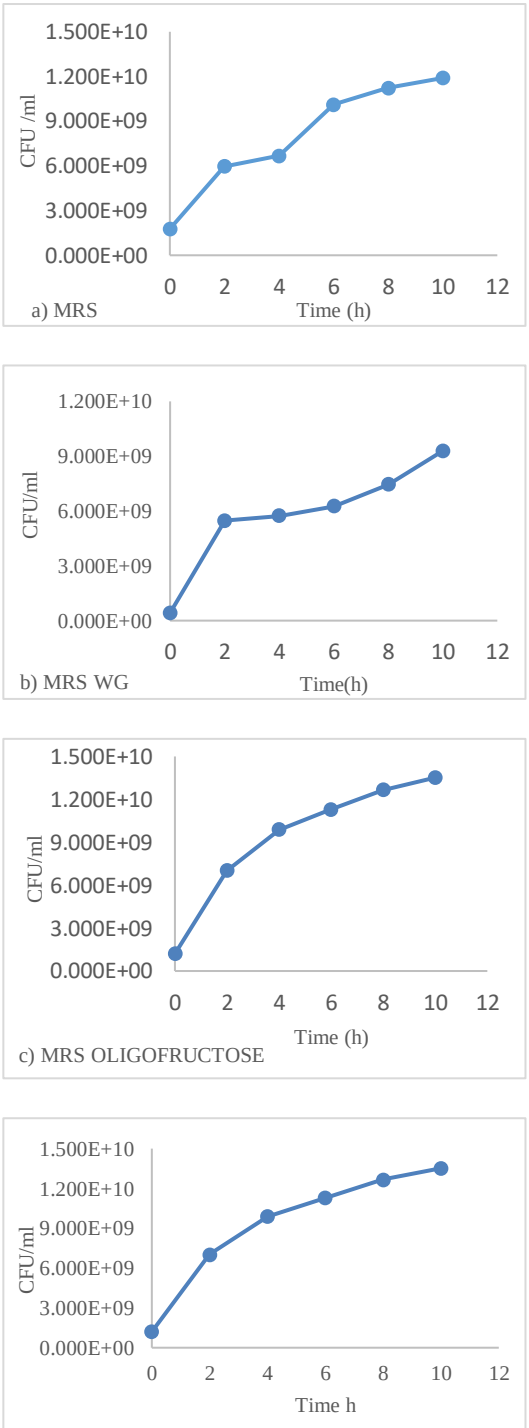

Fig. 5 The growth of the *bacterial* strains in the 4 types of culture media: a)-MRS; b) carbon free MRS;d) MRS chicory inulin;c)- MRS oligofructose

4. Conclusions

This study compared the effect of three carbon substrates in order to increase the biomass of a probiotic culture of *L. plantarum* and *L. rhamnosus*. After 10h incubation, the culture had a significant increase on all three substrates, which led to an improvement in the cultivation of this culture. Further studies will test the viability and degree of encapsulation of probiotics in the most favorable substrates. Since the best encapsulation substrates are carbohydrate polymers with a low polymerization degree [18]-Huang et al., 2017), studies will be conducted on inulin, oligo-fructose and starch by determining the most effective concentrations, degree of encapsulation and viability of probiotics in simulated conditions.

5. Acknowledgement

This work was supported by a grant of the Romania National Council for Higher Education Funding, CNFIS project number CNFIS-FDI-2019-0600.

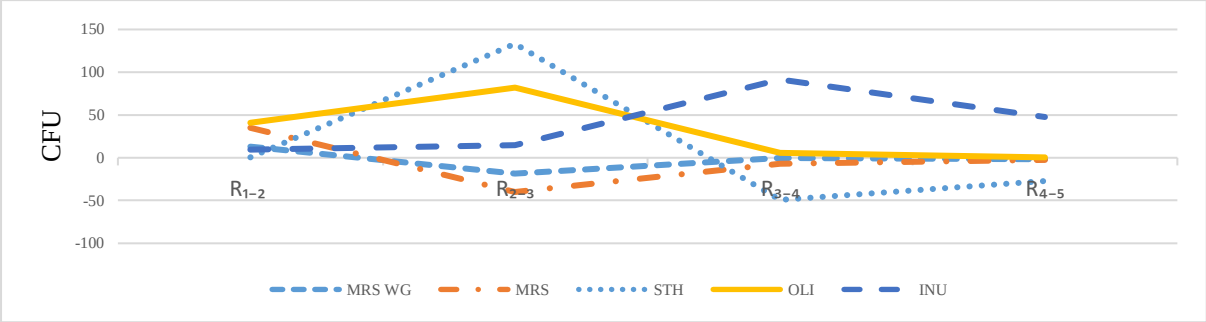

Figure 6 The growth rate of the probiotic culture

Table.1

The pH variation during 10h of monitoring

Media type	time (h)											
	0		2		4		6		8		10	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
MRS STH	5.90	0.012	4.97	0.032	4.65	0.078	4.41	0.006	4.40	0.142	4.36	0.111
MRS OLI	5.95	0.006	5.01	0.012	4.66	0.035	4.49	0.023	4.36	0.006	4.27	0.017
MRS INU	5.90	0.006	5.08	0.017	4.71	0.023	4.51	0.010	4.35	0.021	4.32	0.023
MRS	5.90	0.006	5.16	0.006	5.06	0.006	4.99	0.015	4.81	0.012	4.50	0.006
MRS WG	6.00	0.006	5.12	0.005	4.94	0.006	4.55	0.007	4.43	0.010	4.38	0.006

6. Reference.

[1] de Araújo Etchepare, M., Raddatz, G. C., de Moraes Flores, É. M., Zepka, L. Q., Jacob-Lopes, E., Barin, J. S., ... & de Menezes, C. R. (2016). Effect of resistant starch and chitosan on survival of *Lactobacillus acidophilus* microencapsulated with sodium alginate. LWT-Food Science and Technology, 65, 511-517.

[2]. Ningtyas, D. W., Bhandari, B., Bansal, N., & Prakash, S. (2019). The viability of probiotic

Lactobacillus rhamnosus (non-encapsulated and encapsulated) in functional reduced-fat cream cheese and its textural properties during storage. Food Control, 100, 8-16.

[3]. Castro, J. M., Tornadijo, M. E., Fresno, J. M., & Sandoval, H. (2015). Biocheese: a food probiotic carrier. BioMed research international, 2015

[4]. Wu, Y., & Zhang, G. (2018). Synbiotic encapsulation of probiotic *Latobacillus plantarum* by alginate-arabinoxylan composite microspheres. LWT, 93, 135-141

[5]. Takagi, R., Tsujikawa, Y., Nomoto, R., & Osawa, R. (2014). Comparison of the growth of Lactobacillus delbrueckii, L. paracasei and L. plantarum on inulin in co-culture systems. Bioscience of microbiota, food and health.

[6]. Sathyabama, S., & Vijayabharathi, R. (2014). Co-encapsulation of probiotics with prebiotics on alginate matrix and its effect on viability in simulated gastric environment. LWT-Food Science and Technology, 57(1), 419-425.

[7]. de Melo Pereira, G. V., de Oliveira Coelho, B., Júnior, A. I. M., Thomaz-Soccol, V., & Soccol, C. R. (2018). How to select a probiotic? A review and update of methods and criteria. Biotechnology advances Sánchez,

[8]. Sánchez, M. T., Ruiz, M. A., Lasserrot, A., Hormigo, M., & Morales, M. E. (2017). An improved ionic gelation method to encapsulate Lactobacillus spp. bacteria: Protection, survival and stability study. Food Hydrocolloids, 69, 67-75.

[9]. König, H., Unden, G., & Fröhlich, J. (2009). Biology of Microorganisms on Grapes, in Must and in Wine. Springer.

[10]. Quigley, E. M. (2018). Prebiotics and probiotics in digestive health. Clinical Gastroenterology and Hepatology.

[11]. Lopes, S. M. S., Krausová, G., Carneiro, J. W. P., Gonçalves, J. E., Gonçalves, R. A. C., & de Oliveira, A. J. B. (2017). A new natural source for obtainment of inulin and fructo-oligosaccharides from industrial waste of Stevia rebaudiana Bertoni. Food chemistry, 225, 154-161

[12]. Gänzle, M., & Follador, R. (2012). Metabolism of oligosaccharides and starch in lactobacilli: a review. Frontiers in microbiology, 3, 340.

[13]. Humblot, C., Turpin, W., Chevalier, F., Picq, C., Rochette, I., & Guyot, J. P. (2014). Determination of expression and activity of genes involved in starch metabolism in *Lactobacillus plantarum* A6 during fermentation of a cereal-based gruel. International journal of food microbiology, 185, 103-111

[14]. Roberfroid, M. B., Van Loo, J. A., & Gibson, G. R. (1998). The bifidogenic nature of chicory inulin and its hydrolysis products. The Journal of nutrition, 128(1), 11-19.

[15]. Leenen, C. H., & Dieleman, L. A. (2007). Inulin and oligofructose in chronic inflammatory bowel disease. The Journal of nutrition, 137(11), 2572S-2575S.

[16] Makras, L., Van Acker, G., & De Vuyst, L. (2005). Lactobacillus paracasei subsp. paracasei 8700: 2 degrades inulin-type fructans exhibiting different degrees of polymerization. Appl. Environ. Microbiol., 71(11), 6531-6537.

[17]. Pimentel-González, D. J., Campos-Montiel, R. G., Lobato-Calleros, C., Pedroza-Islas, R., & Vernon-Carter, E. J. (2009). Encapsulation of Lactobacillus rhamnosusin double emulsions formulated with sweet whey as emulsifier and survival in simulated gastrointestinal conditions. Food Research International, 42(2), 292-297

[18]. Huang, S., Vignolles, M. L., Chen, X. D., Le Loir, Y., Jan, G., Schuck, P., & Jeantet, R. (2017). Spray drying of probiotics and other food-grade bacteria: A review. Trends in food science & technology, 63,1