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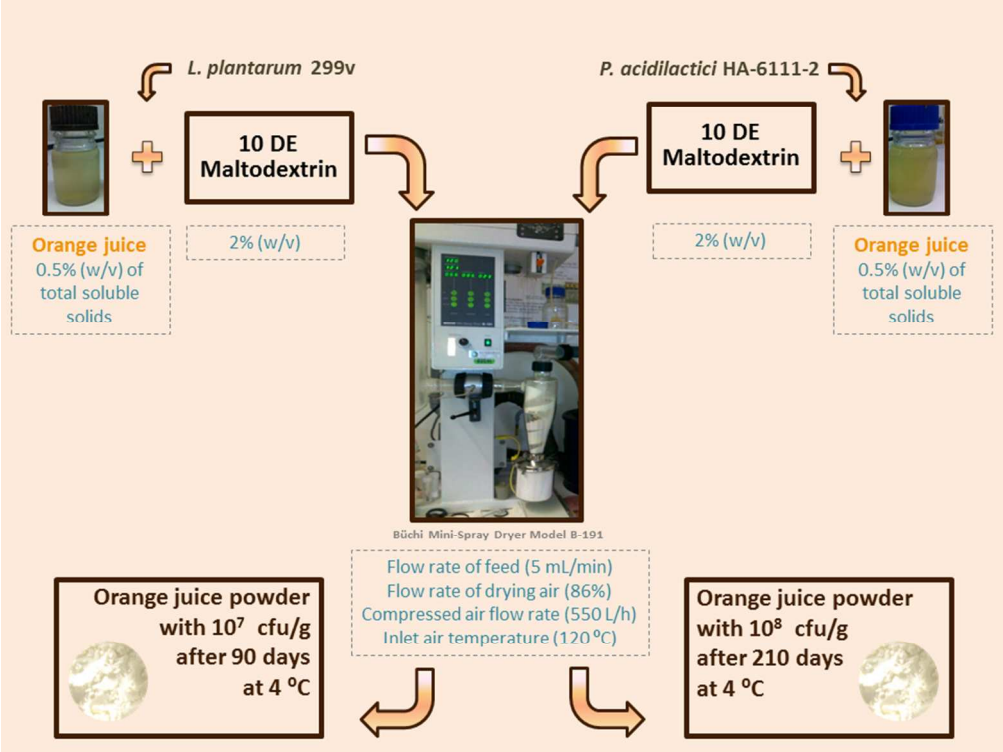

Graphical abstract

254x190mm (96 x 96 DPI)

Spray drying conditions for orange juice incorporated with lactic acid bacteria

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Running title: Probiotic orange juice powder

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Abstract

This work aimed to develop an orange juice powder by spray drying with lactic acid bacteria (*Lactobacillus plantarum* 299v and *Pediococcus acidilactici* HA-6111-2), testing their survival both during drying and storage (room temperature and 4 ºC). Initially, the best conditions for spray drying were chosen to allow the best survival of each LAB: i) inlet air temperature of 120 °C and ii) 0.5:2 ratio of the orange juice soluble solids and drying agent added (prebiotics: 10DE maltodextrin or gum Arabic). Survival of LAB was not affected by drying process and it was higher when cultures were stored at 4 ºC. A slightly higher protection was conferred by 10DE maltodextrin, in the case of *L. plantarum* and at 4 ºC. *Pediococcus acidilactici* was more resistant during storage at 4 ºC, with logarithmic reductions lower than 1 log-unit. It was demonstrated that it is possible to produce a functional non-dairy product, orange juice powder supplemented with prebiotic compounds, containing viable LAB for at least 7 months, when stored at 4 ºC.

Keywords: Spray drying, *Lactobacillus plantarum* 299v, *Pediococcus acidilactici* HA-6111-2, maltodextrin, gum Arabic

Introduction

The production of oranges in Portugal is more than 200,000 tons per year (OMAIAA, 2011) and the possibility of producing natural orange juice powder would be an advantage at economic level, not only by the reduction in volume and weight of the packages, easier transportation and storage, but especially by increasing the shelf life of the product. At the same time, the use of probiotics as food supplements is increasing, because of their health benefits, as well as the increased diversity in food choices they provide. Presently, probiotics are defined as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" by the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO, 2002). Producing an orange juice with probiotic bacteria can be an innovative way to increase this diversity in food choices, especially among consumers who prefer functional non-dairy based foods. Whilst dairy products are the priority for the development of novel probiotic foods, an increase of vegetarianism, milk cholesterol content, and lactose intolerance justify the need for non-dairy probiotic products (Granato *et al.*, 2010).

Spray drying (SD) is the most common method used for converting liquid food products into dry powder, because it is inexpensive and easy to operate. Briefly, the process involves the pumping of liquid sample into the atomizer that transforms the liquid into small droplets, which rapidly lose their moisture on contact with the hot and dry air (Silva *et al.*, 2011). It could be a good method to get a natural orange juice powder, if the characteristics of the natural juice allowed the powder production. Fruit juices are extremely sticky, due to the presence of low molecular weight sugars and organic acids in their composition, their high hygroscopicity, water solubility and low melting point (Bhandari et al., 1997). The usual strategy to spray-dry sticky products is the use of wall materials with high molecular weight. Several authors have been using drying aids to a variety of fruit juices, being maltodextrins and gum Arabic the more common agents used

(Martinelli *et al.*, 2007; Tonon *et al.*, 2010). Maltodextrins are low cost oligosaccharides, made from starch, that have dextrose equivalents (DE) and gum Arabic is a complex heteropolysaccharide and a natural exudate of Acacia tree (Bemiller & Whistler, 1996). Together with their ability as drying agents, several studies have provided evidence that both maltodextrin and gum Arabic also have prebiotic effects (Anekella & Orsat, 2013; Slavin, 2013). Prebiotics are defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health" (Gibson & Roberfroid, 1995).

Studies on SD of fruit juices with probiotic bacteria incorporated are rare (Anekella & Orsat, 2013; Pereira *et al.*, 2014), probably because there are many factors influencing the survival of probiotics before, during and after SD (reviewed by Barbosa *et al*., 2016).

This work aimed to develop an orange juice powder dried by SD and incorporating viable lactic acid bacteria (LAB), ensuring their survival both during drying and storage.

Materials and methods

Origin, growth and storage conditions of LAB isolates

Two LAB were used: *Lactobacillus plantarum* 299v (Probis Probiotika, Lund, Sweden) and *Pediococcus acidilactici* HA-6111-2 deposited in *Escola Superior de Biotecnologia* (ESB) culture collection (Barbosa *et al*., 2015).

Growth and storage of isolates were done according to Barbosa *et al*. (2015).

Conditions of the drying process of orange juice

Materials

Mature oranges exclusively originated in Portugal were randomly purchased from local commercial establishments (Porto, Portugal) and stored at room temperature until used (for no more than 24 h before experiments).

The drying agents used were 10 DE maltodextrin (Sigma, Steinheim, Germany) and gum Arabic (Merck, Darmstadt, Germany).

Orange juice preparation

Oranges were squeezed using a domestic juicer and the juice was filtered in order to eliminate the solids in suspension, preventing the obstruction of the atomizer of the spray dryer. The content of the total soluble solids of the juice was measured using a digital refractometer (model PR-32α (alpha), Brix 0–32%, Atago U.S.A., Inc., WA, U.S.A.) and adjusted to 0.5 or 1% (w/v). The drying agents 10 DE maltodextrin and gum Arabic were added, both at the concentrations of 1 or 2% (w/v), under magnetic stirring at 40 ºC, until complete dissolution.

Spray drying

The drying of orange juice was performed in a laboratory scale Büchi Mini Spray Dryer Model B-191 (Büchi Laboratoriums-Technik, Flawil, Switzerland) with a two-fluid nozzle atomizer with a 1 mm inside diameter and a concurrent drying chamber of 10.5 cm (Barbosa *et al.*, 2015). The inlet air temperatures tested were 120 and 130 ºC. The outlet air temperature cannot be regulated, resulting from a combination of the inlet air temperature, the feed rate, the drying gas flow rate and the solids content of the feed. A single cyclone air separator system was used and the dried powders were collected from the base of the cyclone.

Analysis of powders

Immediately after the SD, drying yield and water activity (a_w) of the dried powders were determined (Barbosa *et al*., 2015).

Spray drying of orange juice with LAB

Orange juice preparation

The orange juice was prepared as described above. After the selected conditions, the total soluble solids content of the juice was adjusted to 0.5% (w/v) and at this solution was added 2% (w/v) of the drying agent (10 DE maltodextrin or gum Arabic), under magnetic stirring at 40 ºC, until complete dissolution.

Preparation of LAB cultures

From Man, Rogosa and Sharpe (MRS) agar incubated at 37 ºC for 24 h, one colony of each LAB isolate was transferred to MRS broth and incubated at the same conditions. For the final inoculum, the last culture was transferred to a new MRS broth (1:100) and incubated at 37 ºC for 24 h to reach stationary phase. Each isolate was harvested by centrifugation (8877 x g, 10 min, 37 ºC; Rotina 35R, Hettich, Germany), washed twice in sterile quarter strength Ringer's solution (Lab M, Bury, United Kingdom) and re-suspended in the same volume of the final solution prepared before (in Orange Juice Preparation).

As control, 10% (w/v) of reconstituted skim milk (RSM) powder (Oxoid, Basingstoke, UK) was used to re-suspend the LAB cultures.

Spray drying and powder analysis

The drying of LAB cultures incorporated either in orange juice or in the RSM was achieved as described above. The drying conditions chosen for both LAB cultures were: feed temperature of

40 ºC, feed flow rate of 5 mL/min; 86% of drying air flow rate; compressed air flow rate of 550 L/h, inlet air temperature of 120 ºC and outlet air temperature of about 65 ºC.

Drying yield and a_w of the dried powders were also determined.

Storage conditions

Dried samples were stored in plastic containers, hermetically sealed in glass flasks, in normal atmosphere (air), in the presence of daylight, at 4 ºC and room temperature.

Enumeration of spray dried LAB cultures

The survival of each microorganism was assessed immediately after SD and at regular intervals throughout storage by rehydration of each dried sample to their initial solids concentration in sterile quarter strength Ringer's solution (Lab M). Each rehydrated sample was homogenized for 1 minute and kept at room temperature for 30 minutes followed by serial decimal dilutions and plated in duplicate for enumeration by the drop count technique (Miles and Misra, 1938) on MRS agar. The enumeration of each microorganism re-suspended in both orange juice or RSM before SD was also performed.

The colonies were counted after incubation at 37 ºC for 48 h and the CFU/mL calculated.

Data analysis

Each experiment was done in duplicate. All calculations were carried out using the software IBM SPSS Statistics (version 22.0, IBM Corporation, Armonk, NY, USA). A significance level of $P < 0.05$ was applied to all statistical procedures.

Statistical analysis

Significant differences in microbial counts of each LAB before and after SD were analyzed using a paired-samples t-test.

Microbial counts were transformed to logarithmic reduction using the equation: **log (N/N_0)**, where N is the microbial cell count at a particular sampling time and N_0 is the microbial cell count after SD. To evaluate the values of the variable log N/N_0 (between the first and the last storage time) among different isolates and used treatments (different drying agents) studied, we applied multivariate models of generalized estimating equations (GEE), with identity as binding function, i.e., it was assumed a linear time course over the months of storage. The GEE are a method that allow analyzing repeated or longitudinal measures, taking into account that the measurements in the same individual over time are correlated. The advantage of this method is that it provides consistent estimates of the parameters associated with covariances of the model, even if the assumed correlation structure would be wrong.

Logistic model

Data from logarithmic reductions of each LAB along storage were adjusted with Logistic model using the equation: **$\log (N/N_0) = - C / (1 + Ae^{-B})$**, where N is the microbial cell count at a particular sampling time, N_0 is the microbial cell count after SD, C is the asymptotic value, which evaluates the tail tendency and B is related to the steepness of the curve (higher values are associated to higher inactivation rates) (Chen, 2007).

Results

Powdered orange juice was initially obtained in a Büchi Mini Spray Dryer at i) constant feed temperature (40 ºC), flow rates of feed (5 mL/min), drying air (86%) and compressed air (550 L/h) and ii) varying inlet air temperatures (120 and 130 ºC) as well as the ratio of total soluble

solids (orange juice; 0.5 or 1% w/v): drying aid (10DE maltodextrin or gum Arabic; 1 or 2% w/v). Powders with different drying yield and a_w values were obtained (data not shown). In table S1 are presented the drying yields and a_w values obtained only for the selected conditions: inlet and outlet air temperatures of 120 ºC and 65 ºC, respectively, and ratio of soluble solids: drying aid of 0.5: 2. For both drying agents, drying yield was close to 50% and values of a_w between 0.3 and 0.4 were obtained for the orange juice powders.

In table 1 are shown the log CFU/ml of *L. plantarum* 299v and *P. acidilactici* HA-6111-2 before and after being spray dried. For each LAB, besides the strong correlation coefficient (>0.9), no significant differences were obtained ($P > 0.05$) between before and after the drying process. Moreover, no logarithmic reductions were obtained after SD for both LAB.

Kinetic parameters for logarithmic reductions of each LAB along 210 days of storage at room temperature and 4 ºC are detailed in Table S2.

The survival of each microorganism during storage at room temperature is presented in Figure 1. For both LAB, survival during storage at room temperature was higher when cells were spray dried in RSM. Although, the reduction of both microorganisms had been lower for RSM initially, both were reduced to values below the level of the detection limit of the enumeration technique after 150 (*L. plantarum* 299v; graph A1) or 210 days of storage (*P. acidilactici* HA-6111-2; graph B1).

In spray dried orange juice, significant differences were obtained between the drying agents used regarding to the survival of LAB during storage period studied ($P < 0.05$). After 11 days of storage in orange juice dried with 10 DE maltodextrin (graph A2) an accentuated reduction in the number of cells of *L. plantarum* 299v occurred, and after 60 days the logarithmic reduction was

to values below the level of the detection limit of the enumeration technique. In orange juice dried with gum Arabic (graph A3), even though the similar behavior, higher reduction in the number of cells of *L. plantarum* 299v occurred initially, compared with orange juice dried with 10 DE maltodextrin.

Despite survival of *P. acidilactici* HA-6111-2 dried in orange juice with 10 DE maltodextrin (graph B2) or gum Arabic (graph B3) had been different along the storage period ($P < 0.05$), in both cases, the reduction in the number of cells became more pronounced after 30 days of storage and values below the level of the detection limit were attained after 120 days of storage.

In Figures 2 and S1 are presented the survival of *L. plantarum* 299v and *P. acidilactici* HA-6111-2, respectively, during storage at 4 ºC. A higher survival was observed during storage at 4 ºC than at room temperature, for both LAB.

In the case of *L. plantarum* 299v, the drying with RSM (Fig 2, graph A4) also conferred a protective effect during storage at 4 °C, comparing with the other drying agents ($P < 0.05$). Significant differences were also found among the additives used ($P < 0.05$). In the orange juice dried with 10 DE maltodextrin (Fig 2, graph A5), there was a 4 log-units reduction in the survival of *L. plantarum* 299v up to 90 days of storage and of 8 log-units until the end of the storage period (210 days). In the presence of gum Arabic (Fig 2, graph A6), after this period of 90 days this reduction was higher than in the presence of 10 DE maltodextrin, reaching values below the detection limit after 210 days (> 9.9 log reduction).

No significant differences were observed in the survival of *P. acidilactici* HA-6111-2 during storage at 4 ºC ($P > 0.05$) for all the SD media investigated, RSM (Fig S1, graph B4) and orange juice supplemented with 10 DE maltodextrin (Fig S1, graph B5) or gum Arabic (Fig S1, graph

B6). At this storage temperature, *P. acidilactici* HA-6111-2 demonstrated a higher survival than *L. plantarum* 299v ($P < 0.05$), showing less than 1 log-unit reduction after 210 days. Orange juice dried with 10 DE maltodextrin conferred a slightly higher protection on the survival of *L. plantarum* 299v during storage at 4 ºC.

Discussion

To develop a new product such as an orange juice powder with functional properties, two LAB were selected to be incorporated: *L. plantarum* 299v - a commercial probiotic and *P. acidilactici* HA-6111-2 an isolated strain from a food matrix and with probiotic characteristics found after preliminary characterization (Barbosa *et al.*, 2015).

Optimization of a drying process is the initial step to gather the best conditions for obtaining a powdered product of good quality. The different parameters evaluated to optimize the drying process of orange juice by SD were i) the content of soluble solids in orange juice, as well as the optimal ratio of drying agents tested, allowing the juice drying with the lowest loss of powder, and ii) the inlet air temperature of the SD. With the different tests carried out, conditions leading to the highest drying yield and product with low a_w were selected: 0.5:2 ratio of the orange juice soluble solids and drying agent added.

Another important parameter in SD is the outlet air temperature. As previously mentioned, in the Büchi Mini Spray Dryer used this is not an adjustable parameter, resulting from the combination of the various parameters such as inlet air temperature, pump and aspirator settings and feed concentration. Several researchers reported that outlet air temperatures above 85 ºC were lethal for probiotic cultures (Gardiner *et al.*, 2000; Corcoran *et al.*, 2004). Since we intended to incorporate probiotics into orange juice and the outlet air temperatures are such an important

parameter in the survival of bacteria during drying, all the conditions selected (Table S1) resulted in low outlet air temperatures (close to 65 ºC) and, simultaneously, high drying yields and low a_w values.

According to Bhandari *et al.* (1997) a total powder recovery of 50% in a laboratory scale spray dryer is considered to be the reference point for a marginally successful drying. From both concentrations of the drying agents tested, the addition of 2% (w/v) to the orange juice allowed a better drying yield than 1% (w/v). This is in agreement with some studies which stated that the increasing of drying agents concentration in fruit juices, also increased the powder yield (Quek *et al.*, 2007; Fazaeli *et al.*, 2012). With the subsequent addition of probiotic cultures to orange juice, it was important that the selected conditions allowed to obtain low a_w values, as water remaining after drying affects the viability of cultures, after the drying process and also during storage (Zayed & Roos, 2004). Moreover, dried products with a_w values below 0.6 are considered microbiologically stable (Quek *et al.*, 2007).

After the selection of SD conditions for orange juice powder production, we proceeded to the incorporation of each LAB to the juice with each drying agent - 10 DE maltodextrin and gum Arabic - and subsequent drying. The survival of LAB was not affected during the drying process ($P > 0.05$). Good survival of probiotics after SD in fruit juices had already been reported (Anekella & Orsat, 2013).

Drying of the selected LAB in RSM was used as a control since it has been demonstrated that it is as an efficient protector, both during drying and also during subsequent storage (Teixeira *et al.*, 1995a; Gardiner *et al.*, 2000; Ananta *et al.*, 2005).

As expected, significant differences were obtained between the temperatures of storage used ($P < 0.05$). Many authors reported the higher survival of spray dried bacteria during storage at low temperatures (Teixeira *et al.*, 1995b; Gardiner *et al.*, 2000; Silva *et al.*, 2002).

Of the drying agents used in this study, 10 DE maltodextrin allowed better results on the survival of *L. plantarum* 299v in comparison with gum Arabic. Other studies have demonstrated the importance of maltodextrin as a drying agent in fruit juices, as well as its protective ability of probiotic cultures during drying and subsequent storage (Anekella & Orsat, 2013; Pereira *et al.*, 2014).

Despite the scarce literature regarding the drying of *L. plantarum* and *P. acidilactici* strains incorporated in fruit juices by SD, many authors have studied the behavior of different strains after SD using maltodextrin as carrier. Lapsiri *et al*. (2012) found high survival rate of *L. plantarum* TISTR 2075 after SD. During storage at different temperatures, survival of spray dried cells was affected by elevated temperatures; while at 25 °C no cells have survived up to 90 days of storage, at 4 °C this strain had a decrease of only 1.62 log CFU/g after 12 months of storage in the absence of light. In the study of Reddy *et al.* (2009), using maltodextrin and nonfat skimmed as carriers, during 60 days of storage, the high temperatures also affected the survival rate of *L. plantarum* and *P. acidilactici* strains tested. At 4 ºC a survival rate of 60% was obtained for both strains and carriers. At 30 ºC, using maltodextrin as carrier, the survival rate decreased to 50% for both strains and using nonfat skimmed, the survival rate decreased to 67% for *L. plantarum* and to 53% for *P. acidilactici*. The authors concluded that maltodextrin is a good substitute of nonfat skimmed.

Although maltodextrin could be a good encapsulating agent during SD, it also acts as a prebiotic, allowing the survival of the cultures (Reddy *et al.*, 2009; Lapsiri *et al.*, 2012; Anekella & Orsat, 2013; Pereira *et al.*, 2014).

In this study, and only focusing the results obtained during storage at 4 ºC, it was possible to obtain spray dried cultures in orange juice, which could survive and remain viable in amounts of 10^7 CFU/ml, over a certain period of time. As expected, different microorganisms had different behaviors: while the orange juice powder contained 10^7 CFU/mL of *L. plantarum* 299v only up to 90 days of storage at 4 °C, the orange juice powder with *P. acidilactici* HA-6111-2 contained 10^8 CFU/mL, at least up to 210 days of storage. This means that the conditions are fulfilled for extra and important experiments being performed, especially in terms of i) storage conditions improvement, ii) validation of results in a larger industrial drier and iii) validation of the ability of the dried probiotic cultures to retain its functional properties. Nonetheless, the present data is promising and allowed to prove that it is possible to produce a functional non-dairy based food, such as an orange juice powder that incorporates probiotic ingredients.

Conclusions

The conditions to obtain orange juice powder by SD were pooled. Another challenge was the incorporation of bacteria with probiotic characteristics and that they were able to survive during drying process and storage. In this study it was demonstrated that it is possible to produce a healthy product, not only for the advantage of using a powder made from natural fruit juice, as well as the beneficial characteristics conferred by a prebiotic and probiotic incorporated. Within various additives which can be used in food industry, the prebiotic maltodextrin turned possible not only to obtain a powder of good quality, but also a higher survival of one of the bacteria incorporated into orange juice, along its storage at 4 °C. If this product is stored and sold at refrigerated conditions it can have a long shelf life, depending on the probiotic used. The potential probiotic *P. acidilactici* HA-6111-2 studied was more resistant than the probiotic *L. plantarum* 299v. The challenge continues to be the preservation of these products at room

temperature, so it is necessary to improve the storage conditions in order to increase their shelf life at the lowest possible cost.

Acknowledgments

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Table 1. Survival of *L. plantarum* 299v and *P. acidilactici* HA-6111-2 before and after SD in RSM and orange juice supplemented with 10DE maltodextrin or gum Arabic

	log CFU/ml					
	L. plantarum 299v			*P. acidilactici* HA-6111-2		
	RSM	**10 DE maltodextrin**	**gum Arabic**	**RSM**	**10 DE maltodextrin**	**gum Arabic**
Before SD	10.0±0.09	10.0±0.53	9.4±0.44	10.7±0.29	9.0±0.07	9.1±0.04
After SD	11.3±0.10	11.2±0.05	9.9±0.70	11.0±0.11	9.1±0.23	9.9±0.15

Figure 1. Logarithmic reduction (●) and Logistic model (—) of *L. plantarum* 299v (**A**) and *P. acidilactici* HA-6111-2 (**B**) incorporated in orange juice or RSM after SD and during 210 days of storage at room temperature: (—●—) control (inoculum in 10% (w/v) of RSM); (—●—) orange juice with 2% of 10 DE maltodextrin and (—●—) orange juice with 2% of gum Arabic. The dotted lines mean that the isolate was reduced to values below the detection limited of the enumeration technique.

Figure 2. Logarithmic reduction (●) and Logistic model (—) of *L. plantarum* 299v (**A**) incorporated in orange juice or RSM after SD and during 210 days of storage at temperature of 4 ºC: (—●—) control (inoculum in 10% (w/v) of RSM); (—●—) orange juice with 2% of 10 DE maltodextrin and (—●—) orange juice with 2% of gum Arabic.

Figure S1. Logarithmic reduction (●) of *P. acidilactici* HA-6111-2 (**B**) incorporated in orange juice or RSM after SD and during 210 days of storage at temperature of 4 ºC: (—●—) control (inoculum in 10% (w/v) of RSM); (—●—) orange juice with 2% of 10 DE maltodextrin and (—●—) orange juice with 2% of gum Arabic. Logistic model was not applied to this data.

Table S2. Formal kinetic parameters for logarithmic reductions of each LAB along storage

Table S1. Yield and a_w of orange juice powder at selected drying conditions

Drying agent	Orange juice (0.5% of total soluble solids)	
	Yield[a]	**a_w[b]**
2% 10DE Maltodextrin	40.90 ± 1.13	0.409 ± 0.004
2% gum Arabic	53.05 ± 1.48	0.321 ± 0.021

[a]The powder yield is represented as the media of percentages of powder obtained ± the standard error of the mean

[b]The a_w is represented as the media of a_w values obtained ± the standard error of the mean

Table S2. Formal kinetic parameters for logarithmic reductions of each LAB along storage

Condition	Parameters	*L. plantarum* 299v		*P. acidilactici* HA-6111-2	
		Room temperature	4 ºC	Room temperature	4 ºC
RSM (control)	A ± IC/2	7.035±2.903	4.056±3.630	10.895±4.057	n/a
	B ± IC/2	0.028±0.007	0.012±0.010	0.025±0.005	
	C ± IC/2	11.987±1.112	5.624±4.754	11.866±1.299	
	R^2	0.963	0.828	0.977	
Orange juice with 2% of 10DE MD	A ± IC/2	15.092±18.495	6.264±4.053	35.947±26.417	n/a
	B ± IC/2	0.357±0.151	0.015±0.011	0.070±0.014	
	C ± IC/2	10.232±0.556	10.344±6.223	9.051±0.322	
	R^2	0.939	0.842	0.990	
Orange juice with 2% of GA	A ± IC/2	25.223±18.131	19.137±13.238	83.619±107.016	n/a
	B ± IC/2	0.456±0.100	0.026±0.009	0.067±0.020	
	C ± IC/2	9.862±0.263	10.557±1.808	10.028±0.502	
	R^2	0.985	0.958	0.983	

Legend: B - related to the steepness of the curve; C - the asymptotic value
n/a – not applicable

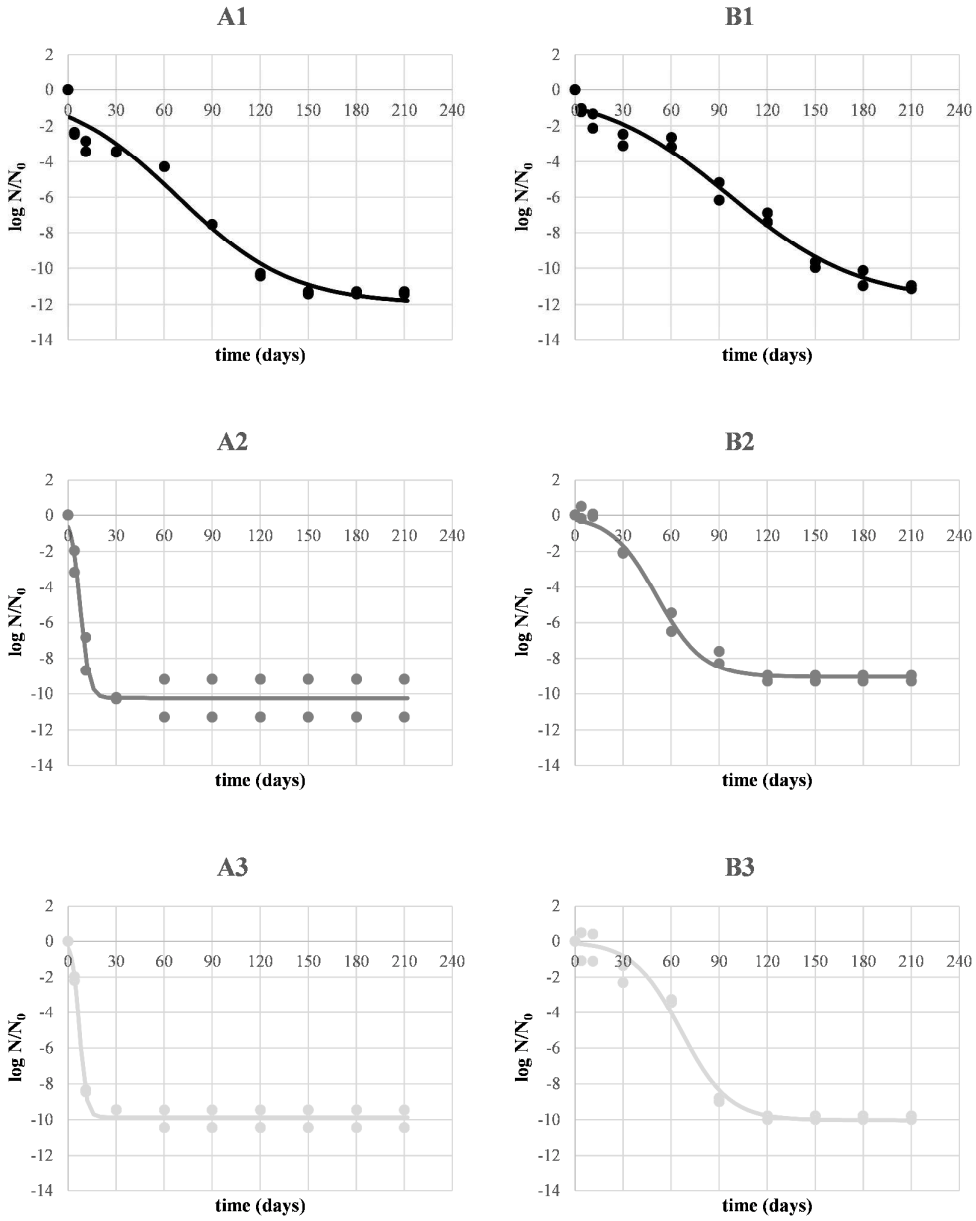
A1
B1
A2
B2
A3
B3
log N/N0
time (days)
2
0
-2
-4
-6
-8
-10
-12
-14
0
30
60
90
120
150
180
210
240

Figure 1.

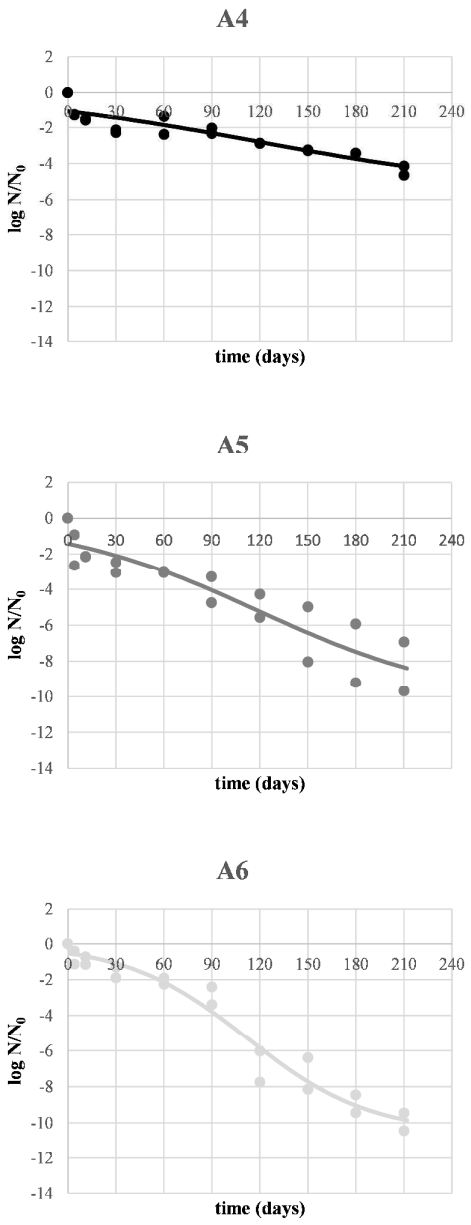

Figure 2.

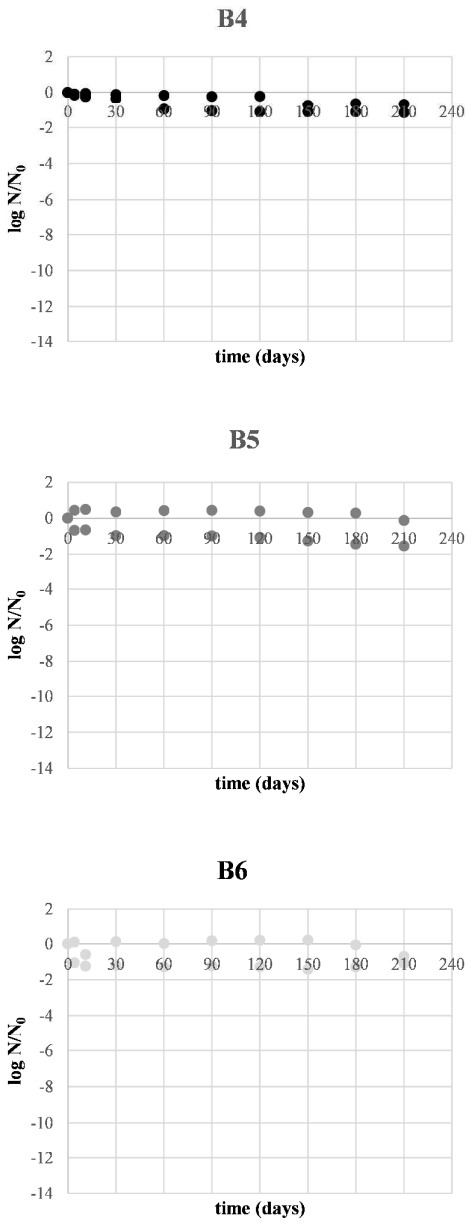
B4
B5
B6
log N/N0
time (days)
2
0
-2
-4
-6
-8
-10
-12
-14
0
30
60
90
120
150
180
210
240

Figure S1.