Improving antioxidant properties and probiotic effect of a citrus juice inoculated with *Lactobacillus salivarius* spp. *salivarius* (CECT 4063) by trehalose addition and/or sublethal homogenization

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Abstract

This study evaluates the effect of trehalose addition (10 or 20%, w/w) and/or sublethal homogenization (25-150 MPa) on antioxidants content (vitamin C, total phenols and flavonoids) and activity (measured both by ABTS-TEAC and DPPH assays), as well as on microbial counts and survival to *in vitro* digestion of clementine juice inoculated with *Lactobacillus salivarius* spp. *salivarius*. Particle size, vacuum impregnation parameters and anti-*Helicobacter pylori* effect were also measured. Incubation with the probiotic improved the antioxidant properties of the juice. Homogenization pressures below 100 MPa following incubation increased both the probiotic counts in the juice and its antioxidants bioaccesibility. Adding 10% (w/w) of trehalose to the juice was effective in preventing these bioactive compounds deterioration under adverse conditions. Once homogenized, liquids containing 10% (w/w) of trehalose became as able as those without trehalose to enter a food solid matrix. Inhibition of *Helicobacter pylori* growth was evident in all probiotic beverages.

Keywords: trehalose, homogenization, probiotic, *in vitro* digestion, antioxidants, anti-*Helicobacter pylori*

1. Introduction

Research carried out in recent years demonstrates the close relationship existing between gut microbiota and the incidence of certain diseases, whether infectious, degenerative, metabolic or psychological (Pirbaglou et al., 2016; Rouxinol-Dias et al., 2016; Siong et al., 2015). There is also evidence of the beneficial effect that food matrices exert on the growth and survival of certain microorganisms during gastric transit, thus conditioning the composition of human microbiota and making probiotics consumption more recommendable as part of a food than in the form of supplements (Ranadheera et al., 2010).

Fermented dairy foods are commonly used as probiotic carriers since they are rich in proteins and lipids that protect them against the adverse conditions of the digestive system (Khan, 2014). However, the consumption of such products is restricted in individuals with lactose intolerance and/or with high cholesterol levels, as well as in the population following vegetarian or vegan diets. This has encouraged the recent use of alternative food matrices for the delivery of probiotics (Anekella & Orsat 2013; Chen & Mustapha, 2012; Furtado-Martins et al., 2013; Rivera-Espinoza & Gallardo-Navarro, 2010). In particular, fruit and vegetable juices have been suggested as an ideal medium for cultivating probiotic microorganisms since they inherently contain beneficial nutrients such as minerals, vitamins, dietary fibber, antioxidants, and they have taste profiles that are pleasing to all the age groups (Rivera-Espinoza & Gallardo-Navarro, 2010). The inherent nutritional value of fruit and vegetable products can be further improved by fermentation with lactic acid bacteria, which is known to promote the production of organic acids, sugar polymers, aromatic compounds, vitamins, polyphenolic compounds, and some useful enzymes, which enrich the human diet (Ronghao et al., 2018). In addition, due to their fast passage through the digestive tract, the viability of probiotic cells in the juices is hardly affected by the harsh acidic environment of stomach (Vijaya-Kumar et al., 2015). However, these food matrices do not always fulfil the pH or the essential amino acids and vitamins required for the optimum growth of most LAB with proven probiotic effect. It is therefore necessary, apart from selecting the appropriate strain for each substrate, adding certain supports (prebiotics, cryoprotectants, soygerm powder, yeast extract, etc.) and/or applying some processing technologies (microencapsulation, vacuum impregnation, sublethal homogenization, etc.) in order to meet the minimum concentration of 10^6-10^7 viable probiotic

cells per millilitre or gram of product at the expiration date, which is required to make an EU-based health claim (Rad et al., 2013). Since benefits attributed to probiotics do not only depend on the amount ingested, it is also important to evaluate how probiotics properties such as their tolerance to severe conditions (including acid and bile conditions), their ability to adhere to intestinal epithelium, their resistance to antibiotics or their antimicrobial activity are affected under such conditions.

Trehalose is a very important natural metabolite and a prospective food additive. This non-reducing sugar is known to protect cell membranes and proteins against abiotic stress caused by heat, exposure to ethanol or high concentration of solutes in dehydration processes (Mansure et al., 1994; Nery et al., 2008; Ohtake & Wang, 2011; Wolkers et al., 2001). Viability of 94% was obtained when 10% (w/v) trehalose was used as compared to a complete loss of viability when no protectant was included in freeze-drying *Lactobacillus acidophilus* (Dodoo et al., 2017). Also, after three months of storage of oral capsules containing freeze-dried *Lactobacillus paracasei* subsp. *tolerance* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, the maximum survival rate (about 72-76%) was observed with media containing 6% (w/v) skim milk, 8% (w/v) trehalose and 4% (w/v) sodium ascorbate (Jalali et al., 2012). In genetically modified *Lactobacillus lactis*, trehalose production not only lead to nearly 100% viability following prolonged storage in a freeze-dried form, but also enhanced viability in human gastric juice without interfering with their therapeutic efficacy in secreting interleukin-10 (Termont et al., 2006). Therefore, trehalose has broad biotechnological applications, especially as a preservative of food and biological products, but also as a sucrose substitute in bakery and pastry products with an almost flat response on blood glucose levels (Ohtake & Wang 2011). Indeed, given that the enzyme required for the body to convert trehalose back into glucose is found in a finite amount in the small intestine, only a small amount of glucose can be produced at any time. In addition, since the development of a novel enzymatic system for its production and its recognition as a safe food ingredient by the EU and the US regulation systems, trehalose is produced on an industrial scale and so it can be used in food formulation without notably increasing the final cost (Higashiyama, 2002; Schiraldi et al., 2002).

High pressure homogenization (HPH) is a unit operation traditionally employed in juice manufacturing as an alternative to thermal treatment for microbial inactivation. According to

recent studies, this new technology has been proven to enhance the survival and the overall functionality of certain probiotic strains when applied at moderate levels (Tabanelli et al., 2012). In particular, sublethal HPH has been reported to enhance organoleptic and functional properties of probiotic fermented milks and cheeses by improving strain viability over refrigerated storage and accelerating fermentation kinetics (Burns et al., 2015; Patrigniani et al., 2009). Lanciotti et al. (2007) also showed that HPH not exceeding 150 MPa was able to modify both the fermentation kinetics and the enzymatic activities of starter and non-starter lactic acid bacteria without detrimental effects on cell viability. When applied to low pulp mandarin juice, the homogenization in the range between 5 and 30 MPa significantly reduced the average size of suspended solids without negatively affecting the antiradical activity and the content of bioactive compounds (Betoret et al., 2012a). Therefore, when using the homogenized juice as impregnating solution, more liquid and more functional compounds are expected to be introduced into the structural matrix of the impregnated sample. Beyond these effects of mainly technological interest, non-lethal HPH was observed to increase the hydrophobicity in trials with different probiotic strains, which is directly related to their capacity of adhesion to the intestinal cells and their resistance to the digestion process (Basson et al., 2007; Betoret et al., 2017; Tabanelli et al., 2012). Also recent studies have evaluated the potential effect of high pressure homogenization for the microencapsulation of probiotic lactic acid bacteria. As reported by Patrignani et al. (2017), a treatment of 50 MPa for 5 passes resulted in stable and homogeneous microcapsules of *Lactobacillus paracasei* A13 and *Lactobacillus salivarius* subsp. *salivarius* CET 4063 that, when used to produce fermented milk, decreased the hyperacidity phenomena and are were more resistant to the simulated digestion process. Microencapsulation with alginate by homogenization at 70 MPa for 2 passes also seemed to be a promising strategy to protect *Lactobacillus salivarius* spp. *salivarius* during gastrointestinal digestion and storage of low pulp mandarin juice (Calabuig-Jiménez et al., 2019).

According to everything commented above, the present study aims to evaluate the effect of trehalose addition (10 or 20% by weight) and/or sublethal homogenization (25, 50, 100 or 150 MPa) on some physicochemical (water activity, Brix, pH, particle size, vacuum impregnation parameters and main antioxidants content and activity) and microbial properties (microbial

counts, survival to *in vitro* digestion and anti-*Helicobacter pylori* effect) of clementine juice inoculated with *Lactobacillus salivarius* spp. *salivarius* CECT 4063.

2. Materials and methods

2.1. Raw materials and bacterial cultures

To carry out this study, Granny Smith variety apples, commercial clementine juice (Hacendado trademark) and food-grade trehalose obtained from tapioca starch (TREHA™, Cargill Ibérica, Barcelona, Spain) were employed. *Lactobacillus salivarius* spp. *salivarius* strain CECT 4063 and *Helycobacter pylori* strain NCTC 11637 also used in this research were supplied by the Spanish Culture Type Collection (Paterna, España) and the National Collection of Type Cultures (Public Health England, UK), respectively. Stock cultures were prepared by adding sterile glycerol (20% v/v) to the activated culture and freezing at -20 ℃ in sterile screwcap cryovials. When necessary, glycerol was removed from the thawed stock culture by centrifugation at 3000 rpm and 4 ℃ for 1 min. Then, probiotic stock cultures were subcultured onto Man Rogosa and Sharp broth (Scharlab, S.L., Barcelona, Spain), while pathogenic stock cultures were plated on Columbia Agar Base supplemented with 10% (v/v) of defibrinated horse blood (Scharlab, S.L., Barcelona, Spain). After 24 h of incubation at 37 ℃ under anaerobic conditions using AnaeroGen sachets (Oxoid Ltd., Basingstoke, UK), a probiotic cell density of $(2.8 \pm 0.8) \times 10^9$ CFU/mL was reached. In the case of *Helicobacter pylori*, total biomass obtained after 48 h of incubation at 37 ℃ under anaerobic conditions using AnaeroGen sachets (Oxoid Ltd., Basingstoke, UK) was resuspended in Brucella broth (Scharlab, S.L., Barcelona, Spain) until reaching a concentration around 10^9 CFU/mL, corresponding to a value of 6 on the McFarland optical density scale.

2.2. Probiotic juices preparation and homogenization procedure

According to the procedure described by Betoret et al. (2012b), commercial clementine juice pH was increased to 6.5 by adding around 9.8 g/L of sodium bicarbonate. In addition, in order to

promote the microbial growth, 5 g of freeze-dried yeast extract were incorporated per liter of juice. When necessary, trehalose was added at a rate of 10 or 20 grams per 100 grams of juice prior to its inoculation with *Lactobacillus salivarius* spp. *salivarius* to get an initial concentration of 7.03 ± 0.13 log CFU/mL. After incubation for 24 h at 37 ºC to achieve the stationary phase, part of the juice-based probiotic liquids were submitted to homogenization at 25, 50, 100 or 150 MPa in a laboratory scale high pressure homogenizer (Panda Plus 2000, GEA-Niro Soavi, Parma, Italy). Homogenized and non-homogenized (0 MPa) juices were then kept refrigerated at 4 ºC until analysis.

2.3. Analytical determinations

All the analytical determinations described in this section were performed at least in triplicate and, depending on the variable being analyzed, within 24 h after the incubation and/or the homogenization step.

2.3.1. a_w, Brix, pH, particle size and vacuum impregnation parameters

Water activity (a_w) was measured with an accuracy of ± 0.003 in a dew point hygrometer (Decagon Agualab, model CX-2), previously calibrated with reference saturated salts. Total soluble solids content expressed in Brix was measured in a table refractometer (Abbe Atago, model Nar-T3) at the constant temperature of 20 ºC. The pH was measured in a potentiometer provided with a temperature self-calibrating system (Mettler Toledo, model S20 SevenEasy™), previously calibrated with buffer solutions at pH 4 and 7. The size of the particles was analyzed in a Malvern Mastersizer analyzer (Malvern Instruments Ltd, model 2000) in a measuring range between 0.02 and 1000 microns. Values employed for the refractive cloud and the dispersed phase indices were 1.73 and 1.33 respectively, while that set for the absorption of the cloud particles index was 0.1 (Corredig et al., 2001). Apart from the corresponding distribution curves of the particle size, also the mean diameter over volume (D[4,3]) and the particle diameter at 90% in the cumulative distribution (d_{90}) were obtained for each sample. Finally, the ability of the different juices to be employed as impregnating solutions in a vacuum impregnation process

was analysed. For this purpose, vacuum impregnation trials were conducted in a pilot plant equipment specially designed for taking the weight measures necessary for the characteristic impregnation parameters calculation (Salvatori et al., 1998): the volume fraction of the solid matrix that was filled with the impregnating solution after the vacuum stage (X_1, in m^3 solution/m^3 fresh sample) and that after the atmospheric stage (X, in m^3 solution/m^3 fresh sample); the relative volumetric deformation undergone by the solid matrix after the vacuum step (γ_1, dimensionless) and that after the atmospheric step (γ, dimensionless); and the effective porosity (ε, dimensionless). Granny Smith apple rings (65 mm outer diameter, 20 mm inner diameter and 5 mm thick) were employed as solid matrix. The working conditions were set at 50 mbar for 10 min and atmospheric pressure for 10 min more.

2.3.2. Antioxidant properties

Vitamin C content was determined by potentiometric titration with a 0.005 M chloramine-T solution and a platinum electrode (Metrohm 800 dosino Ti Application note nº T30). Sample preparation was performed by mixing 15 mL of juice with 50 mL of distilled water, 2 mL of sulfuric acid 2 M and 10 mg of potassium iodide. A 350 ppm ascorbic solution was used for standardizing the titrant solution. Results were expressed in mg of ascorbic acid per mL (mg AA/mL).

Total phenols content was determined by the Folin-Ciocalteu method (Singleton & Rossi, 1965), which is based on measuring at 760 nm the intensity of the blue color that appears by reaction at basic pH between the Folin-Ciocalteu reagent (mixture of phosphor wolframic and phosphor molybdic acids) and those phenols present in the sample. For carrying out measurements, 125 μL of sample diluted in bidistilled water at a ratio 1:5 (v/v) were mixed in a spectrophotometer cuvette with 125 μL of the Folin-Ciocalteu reagent and 500 μL of bidistilled water. After 6 min in darkness, 1.25 mL of a 7.5% (w/v) sodium carbonate solution and 1 mL of bidistilled water were added. Absorbance was measured after 90 min of reaction at 760 nm in a Thermo Scientific Helios Zeta UV/Vis spectrophotometer. A white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. Results

obtained were compared with a gallic acid standard and expressed in mg of gallic acid equivalents per mL of sample (mg GAE/mL).

Total flavonoids content was determined by the colorimetric method proposed by Luximon-Ramma et al. (2005). In this case, 1.5 mL of sample diluted in bidistilled water at a ratio 1:5 (v/v) were mixed in a spectrophotometer cuvette with 1.5 mL of a 2% (w/v) in methanol aluminum chloride solution. Absorbance was measured after 10 min of reaction in darkness at 368 nm in a Thermo Scientific Helios Zeta UV/Vis spectrophotometer. A white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. Results obtained were compared with a quercetin standard and expressed in mg of quercetin equivalents per mL of sample (mg QE/mL).

Antioxidant capacity of juice samples was analyzed both by the ABTS-TEAC and the DPPH assays. ABTS-TEAC assay is based on measuring ABTS$^-$ cation discoloration from green-blue to colorless when reduced by the antioxidant compounds of the sample. In order to produce the ABTS$^-$ cation, a solution containing 7 mM of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and 2.45 mM of potassium persulfate in bidistilled water was incubated in darkness for 16 h. Once the radical was released, it was diluted in phosphate buffer until an absorbance of 0.7 ± 0.01 at 734 nm was reached (Re et al., 1999). Then, 2910 μL of this solution was mixed with 90 μL of diluted sample (1 mL of juice in 9 mL of bidistilled water). Absorbance was in this case measured after 6 min of reaction in darkness at 734 nm in a Thermo Scientific Helios Zeta UV/Vis spectrophotometer. A white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. Results obtained were compared with a trolox standard and expressed in mg of trolox equivalents per mL of sample (mg TE/mL).

DPPH assay measures the color change from purple to yellow that takes place when a solution containing radical DPPH (2,2-diphenyl-1-picrylhydrazyl) is reduced by the antioxidant compounds of the sample (Brand-Williams et al.,1995; Molyneux, 2004). To achieve this, 100 μL of diluted sample (1 mL of juice in 4 mL of distilled water), 900 μL of spectrophotometric grade methanol (purity ≥ 99%) and 2000 μL of a 100 mM solution of DPPH in methanol were mixed in a spectrophotometer cuvette. Absorbance was measured after 30 min of reaction in darkness at 517 nm in a Thermo Scientific Helios Zeta UV/Vis spectrophotometer. As previously mentioned, a white reference sample was prepared by replacing the volume of sample by the

same volume of bidistilled water. Results obtained were compared with a DPPH standard and, according to the initial amount of DPPH poured into the cuvette, expressed in mg of reduced DPPH per mL of sample (mg DPPH$_{red}$/mL).

2.3.3. Microbial counts

Lactobacillus salivarius spp. *salivarius* content in inoculated juices was estimated by the serial dilution in sterile peptone water and plating procedure. As reported in Collado & Hernández (2007), Lactobacilli MRS Agar plates were incubated at 37 ºC for 2 days under anaerobic conditions using AnaeroGen sachets (Oxoid Ltd., Basingstoke, UK).

2.3.4. *In vitro* gastrointestinal simulation

In order to evaluate the potential probiotic resistance to the intestinal and gastric juices, a modification of the protocol proposed by García-Hernández et al. (2018) for *in vitro* simulation of the gastrointestinal process was employed. To simulate gastric digestion stage, a sterile saline solution (0.5%, w/v) containing 3 g of pepsin isolated from porcine gastric mucosa (Sigma Life Science) and adjusted to pH 2 with 0.5 N hydrochloric acid was employed. To simulate intestinal digestion stage, a sterile saline solution (0.5%, w/v) containing 1 g of pancreatin isolated from porcine pancreas (Sigma Life Science) adjusted to pH 8 with 0.1 N NaOH solution was used. Since the analyzed samples had no fat in its composition, no bile salts were included in the intestinal simulation stage. Throughout all the process, samples were stirred at 100 rpm in an orbital incubator (Ivymen System) placed inside an incubation oven at 37 ºC. In the first place, 20 mL of juice were mixed with 70 mL of pepsin solution in an Erlenmeyer flask. Microbial counts were performed at different times throughout the process (0, 10, 30, 60 y 120 min) by the dilution and plating procedure described above. Then, 56 mL of the residual liquid obtained after the gastric stage were mixed with 30 mL of pancreatin solution. In this case, microbial counts were performed at the beginning and at 30, 60, 120, 240 y 360 min from the start of the stage by the dilution and plating procedure describe above. The viabilities of *Lactobacillus salivarius* spp. *salivarius* after both the gastric step (%VIAB$_{ST1}$) and the intestinal step

($\%VIAB_{ST2}$) were calculated as the ratio in percentage between the number of living cells leaving and entering each step. In accordance with this, the probiotic resistance to the entire simulated gastrointestinal digestion ($\%VIAB_{TOTAL}$) was calculated from the product of probiotic viabilities obtained for each of the two steps making up the process (García-Hernández et al., 2012).

2.3.5. Anti-*Helicobacter pylori* effect

The inhibitory effect of *Lactobacillus salivarius* spp. *salivarius* strain CECT 4063 on the growth of *Helicobacter pylori* strain NCTC 11637 was tested by an agar diffusion method, similar to that described by Lin et al. (2011). To this end, aliquots of 100 μL of the pathogen dilution corresponding to a value of 6 on the McFarland optical density scale were spread on Columbia Agar Base plates supplemented with 10% (v/v) of defibrinated horse blood (Scharlab, S.L., Barcelona, Spain). Wells (8 mm in diameter) were punched in each agar plate using a sterile stainless steel borer. Discs of the same size obtained from MRS agar plates seeded with 1 mL of a particular probiotic juice and incubated at 37 ºC for 24 h under anaerobic conditions were place into the wells. Discs obtained by the same procedure from MRS agar plates seeded with 1 mL of the MRS broth with a probiotic cell density of $(2.8 \pm 0.8) \times 10^9$ CFU/mL were used as control. The plates were then incubated at 37 ºC for 48 h under microaerophilic conditions. After that, the diameters of the inhibition zones around the wells were measured. Results were expressed as the mean diameter of triplicate independent experiments for each sample.

2.3.6. Statistical analysis

The statistical significance degree of the different variables considered on the results obtained was evaluated with the Statgraphics Centurion XVI program by means of simple and multivariate analysis of variance with a 95% of confidence level.

3. Results and discussion

3.1. Effect of processing variables on a_w, pH, Brix and particle size of clementine juice

Main physicochemical properties of the different liquids prepared from commercial clementine juice are shown in Table 1. Comparing data obtained for those juices only formulated with sodium bicarbonate and trehalose before their inoculation with the probiotic microorganism (NI samples), the effect of such disaccharide concentration have been evidenced. As expected, increasing the trehalose content from 0 to 20% (w/w) resulted in significant changes in Brix, pH and water activity values but hardly affected the size of the particles present in the juice. In particular, Brix values of non-inoculated liquids significantly increased as it did the amount of trehalose present in the juice, thus reducing the water activity values and promoting osmotic stress with negative effect on microbial growth. Finally, it should be mentioned that, since the disaccharide concentration in the juice was considerably below its maximum solubility in water at 20 ºC (reported in 2009 by Jain & Roy to be 68,9 g/100 g), neither the mean diameter over volume (D[4,3]) nor the particle diameter at 90% in the cumulative distribution (d_{90}) were notably affected by the addition of trehalose (Fig. 1a). The mean diameter over volume was around 223 ± 13 μm and the size of the 90% of the particles present in the juice was of the order of 560 ± 29 μm.

Also from values reported in Table 1 it is possible to realize the effect of the juice incubation with the probiotic microorganism on its main physicochemical properties. What is more outstanding is the sharp decline in pH values, and to a lesser extent in the soluble solids content, as a result of lactic acid production by *Lactobacillus salivarius* spp. *salivarius* CECT 4063 from the available nutrients. Because, as it will be discussed below, the growth of the selected microorganism is adversely affected by increasing the osmolality of the growing media, the pH drop linked to 24 h incubation was less marked as it increased from 0 to 20% by weight the concentration of trehalose in the juice. None of the parameters related to the particles size were noticeably modified by the probiotic growth in the juice, but yes as a result of the application of sub lethal homogenization pressures. According to data shown in Table 1, the homogenization in the range of pressures between 25 and 150 MPa caused, regardless of the pressure applied, a significant decrease in the size of the particles present in the juice. This reduction in both the mean diameter over volume (D[4,3]) and the particle diameter at 90% in

the cumulative distribution (d_{90}) is confirmed in the corresponding distribution curves for particle size (Fig. 1b). As it can be observed, all the analyzed liquids showed a bimodal distribution between 1 and 1000 μm before homogenization (irrespective of having been previously inoculated or not) and between 1 and 200 μm after homogenization. This considerable decrease in the size of the suspended particles resulting from the homogenization step would imply, as reported by Betoret et al. (2012a), an increase in the cloud stability and in the juice incorporation into a food matrix by means of vacuum impregnation without negatively affecting its antiradical activity.

3.2. Effect of processing variables on *Lactobacillus salivarius* spp. *salivarius* CECT 4063 growing in clementine juice

Fig. 2 shows the effect that the trehalose concentration (from 0 to 20 g/100 g) and/or the homogenization pressure (from 0 to 150 MPa) had on the microbial growth of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 in clementine juice at pH 6.5. It should first be noted that incubation resulted in a general rise in the microbial population from $(1.1 \pm 0.3) \times 10^7$ CFU/mL to at least $(1.8 \pm 0.5) \times 10^8$ CFU/mL, which increased even further after homogenization. Therefore, all liquids showed a microbial content over 10^7 CFU/mL, which is the limit established at the time of consumption by the International Dairy Federation to state that a food has probiotic properties (Manojlović et al., 2010).

Regarding the effect of the above-mentioned variables, multifactor analysis of variance showed, at the 95% confidence level, that both the homogenization pressure and the trehalose concentration, as well as the interaction between them, significantly affected the probiotic viability (Fig. 2b). For not homogenized juices (0 MPa), the increase in the trehalose concentration from 0 to 20% (w/w) significantly diminished the number of viable cells. Certainly, as evidenced in Table 1, the lower water activity achieved by the juice as the trehalose concentration increased might induce osmotic stress situations that restricted the growth of the probiotic microorganism. However, differences in the probiotic content due to the amount of disaccharide added to the juice were mildly attenuated by the application of a homogenization step. As evidenced in Fig. 2, microbial counts for a given trehalose content increased with the

homogenization pressure up to a maximum around 50 MPa, when the counts started to decline. This result is comparable to that obtained by Tabanelli et al. (2013) on different lactic acid bacteria strains commonly used in commercial dairy products when submitted to sublethal high pressure homogenization treatments. In his study, Tabanelli et al. (2013) verified that the viability of *L. paracasei* A13, *L. acidophilus* 08 and Dru, *L. delbrueckii* subsp. *lactis* 200 and their bile-resistant derivatives *L. acidophilus* Dru+ and *L. delbrueckii* subsp. *lactis* 200+ suspended in PBS was reduced in less than 0.2 Log CFU/mL by the homogenization at 50 MPa. The increase in the number of living cells observed in the present study when homogenizing in the range 0-50 MPa would highlight the relevance of the matrix effect, so that the viability of *Lactobacillus salivarius* spp. *salivarius* was enhanced in the presence of clementine juice. This finding was previously supported by other authors (Chaikham, 2015; Sagdic et al., 2012; Shah et al., 2010), who attributed to the high content in antioxidant compounds of certain products, as it is the case of clementine juice, the ability to create a favorable anaerobic environment for probiotic survival. Also, adding trehalose to the juice involved slight increase in the viable counts after homogenization at 50 MPa, thus evidencing its role in preserving proteins and biological membranes under moderate stressing conditions (Atarés et al., 2009; Betoret et al., 2014; Crowe et al., 1984; Lins et al., 2004). However, the increase in the trehalose concentration from 0 to 20 g/100 g had just the opposite effect when homogenization pressures above 50 MPa were applied. In other words, the beneficial effect that trehalose had on the probiotic viability was blinded by the adverse effect of the osmotic stress when coupled with high pressure gradients. Thus, in order to achieve the greatest number of living cells in clementine juice it would be advisable to apply homogenization pressures and trehalose concentrations not exceeding 100 MPa and 10% by weight, respectively.

3.3. Effect of processing variables on antioxidant properties of clementine juice

The effect of the formulation with 10% of trehalose by weight, the inoculation and incubation with *Lactobacillus salivarius* spp. *salivarius* and the homogenization at 50 or 100 MPa on main antioxidant properties analyzed in the different liquids obtained from commercial clementine juice is shown in Table 2.

Starting with the ascorbic acid or the vitamin C, none of the variables considered caused significant changes in its content (p-value < 0.05). Despite being a commercial juice that may have been submitted to intense thermal processing, vitamin C content was similar to that reported in previous studies for freshly squeezed clementine juice (Bermejo & Cano, 2012; Fabroni et al., 2016). This result suggests that the juice was probably enriched with this vitamin. In any case, both natural and/or added ascorbic acid remained unchanged against the slight temperature increase (observed to be between 17 and 21 ºC per 100 MPa) caused by forcing the liquid to pass through a very tight hole.

Regarding the total phenols and flavonoids content (Table 2), they behaved in a similar way against variations in the processing conditions, which is logical given that flavonoids are a particular type of phenols.

In the case of non-inoculated juices, the addition of 10 g of trehalose per 100 g of juice resulted in a significant decrease in both total phenols and flavonoids content. This effect was previously observed by Oku et al. (2005) for unsaturated fatty acids and explained in terms of trehalose ability to prevent oxidation by joining double bounds of the acyl chains, which are also part of the chemical structure of the most abundant flavonoids (hesperidin, narirutin and didymin) in clementine juice (Betoret et al., 2009). Therefore, the decrease in the antioxidant compounds of phenolic type content reported after the addition of trehalose to the juice composition could be due to the fact that they were more protected against the oxidation induced for their quantification.

Regarding the growth of the probiotic bacteria in the juice, it significantly increased both total phenols but specifically flavonoids content. This potential of lactic-acid fermentation to release bound phenolic compounds or to convert phenolic compounds into different metabolites, which can exert other bioactivities, is in accordance to that observed in previous studies over different food matrices. Hole et al. (2012) reported a 20-fold increase in the content of total free phenolic acids in both barley and oat flour fermented with different food-graded lactic acid bacteria. Also enhancing the release of phenolic acids (gallic acid) and flavanols (catechin and epicatechin) was reported after soybean fermentation with *Bacillus pumilus* (Cho et al., 2011) and *Bacillus subtilis* (Chung et al., 2011). Both spontaneous but mainly induced by *Lactobacillus plantarum* fermentation of cowpea flour gave rise to some phenolic compounds not detected in the raw

flour, such as tyrosol and quercetin, but a decrease in most hydroxycinnamic derivatives (Dueñas et al., 2005). In a food matrix closer to the one analyzed in the present study, such as whole apple juice fermented with *Lactobacillus acidophilus*, the content of certain phenolic compounds increased or decreased depending on the fermentation time, the pH of the media and the apple variety (Ankolekar et al., 2012). In general, gallic acid content increased and catechin content decreased after 24 h of incubation in samples adjusted to pH 6, but the trend for epicatechin and quercetin derivatives content was different depending on the apple variety. A stated by Huynh et al. (2014), this change in the profile of phenolic compounds by the fermentation process is due to the action of cellulolytic, ligninolytic and pectinolytic enzymes, mainly activated by the pH lowering caused by the growth of the microorganisms. This suggests that lactic-acid bacteria would increase the bioavailability of certain phenolic compounds.

In relation to the subsequent homogenization of the incubated juice, its effect on phenols and flavonoids total content was slightly different, depending on the presence or absence of trehalose in the juice. Therefore, when 10% of threalose by weight was added to the juice the homogenization caused, regardless of the pressure applied, a slight but significant increase in both total phenols and flavonoids content. Since phenolic compounds are known to be bounded to the juice pulp fraction, when its average size was reduced by the application of a homogenization step, total phenols contained therein would be expected to release to increase their bioavailability. However, when no trehalose was added to the juices, such phenolics release seemed to favor their faster degradation.

Finally, antioxidant activity response to the processing variables considered was different depending on the analytical method employed (Table 2). As reported by some authors (Stratil et al., 2007), the DPPH assay could be less selective than the ABTS one since the DPPH radical is only able to react with the most reactive phenols and not with less reactive and more stable ones. What is observed in the present study is that the antioxidant activity measured by the ABTS-TEAC assay was mainly affected by the microbial growing, whereas that measured by the DPPH assay was more affected by the addition of trehalose or the application of a homogenization step. Common for measurements obtained by the two different assays was the more or less evident decline in the antioxidant activity values after the homogenization at 50 or 100 MPa, especially in inoculated juices containing 10% of trehalose by weight.

3.4. Effect of processing variables on impregnating properties of clementine juice

The values of the main parameters obtained after the vacuum impregnation experiments carried out at pilot scale with 5 mm thick apple rings and the different liquids obtained from commercial clementine juice are shown in Table 3. It is worth noting that the average volumetric impregnation parameter value (X = 0.2 ± 0.04 m³/m³) was similar to that obtained in previous studies using sucrose isotonic solutions (Fito et al., 2001) and apple juice or whole milk inoculated with *L. casei* spp. *rhamnosus* (Betoret et al., 2003) as impregnating liquids.

Statistical analysis of the results showed, with a 95% confidence level, a significant effect of both the processing variables (the addition of 10% of trehalose by weight and the homogenization at 50 or 100 MPa) and the interaction between them. In general terms, the addition of 10 g of trehalose per 100 g of clementine juice caused a reduction in the apple rings volume (negative values of the impregnating parameter γ), but this slightly increased after the vacuum impregnation with free from trehalose liquids. This result seems logical considering that trehalose increases the solution hypertonicity, so that osmotic dehydration mechanisms appear coupled to the hydrodynamic ones due to the pressure gradients imposed to the system. For this reason, in addition to the possibly higher viscosity of the impregnating solutions, both the volume of apple that was filled with the impregnating liquid (X) and the solid matrix effective porosity (ε) noticeably decreased as a result of the addition of trehalose. However, due to the significant reduction in the average size of the particles present in the impregnating solution, these two impregnating parameters increased significantly after the homogenization at either 50 or 100 MPa.

From both the liquid inlet (X in m³ liquid/m³ fresh apple) and composition (yⁱ in CFU or mg i/mL liquid), the amount of each functional compound that is incorporated during the vacuum impregnation step per gram of fresh apple (m^i) can be calculated by assuming that neither generation nor degeneration of biocompounds occurred (Eq. 1).

$$m^i = X \frac{y^i}{\rho_a}$$

Eq. 1

where ρ_a is the apparent density of fresh apple, considered to be 0.802 g/mL (Fito et al., 2001).

According to the results shown in Table 4, it should be more recommendable using clementine juice containing 10% of trehalose by weight and submitted to homogenization at 50 MPa as impregnating liquid in order to achieve the highest content of antioxidant compounds in the vacuum impregnated product. However, in order to also reach a great number of living cells in the impregnated apples, the vacuum impregnation with any of the liquids 0%TREH_50MPa or 0%TREH_100 MPa would be the best option.

3.5. Effect of processing variables on *Lactobacillus salivarius* spp. *salivarius* resistance to *in vitro* digestion

Probiotic survival during each step of the *in vitro* digestion (the gastric stage in acid media and the intestinal stage in basic media), as well as during the entire digestion process, is shown in Table 5.

Values obtained after the whole gastrointestinal simulation were of the same order as those reported for other lactic acid bacteria, such as *Lactobacillus delbrueckii* spp. *bulgaricus* and *Streptococcus thermophilus* inoculated in a lactic substrate, with mean cumulative viabilities around 26.2% and 9.2%, respectively (García-Hernández et al., 2012), or *Lactobacillus reuteri* inoculated in raw and fried tomato, with mean cumulative viabilities around 24% and 26.3%, respectively (García-Hernández et al., 2018).

As expected, *Lactobacillus salivarius* spp. *salivarius* CECT 4063 was found to be more resistant to the basic pancreatin solution emulating bowel conditions than to the acid pepsin solution emulating gastric juices secreted in the stomach ($\%VIAB_{ST2} > \%VIAB_{ST1}$). Since the microbial counts were observed to slightly decrease along both the gastric and the intestinal step (Fig. 3), the probiotic bacteria difficulties in adapting to extreme acidic conditions at the beginning of the digestion process were assumed to be the main responsible for their loss of viability. However, when the probiotic bacteria were already stressed by the addition of 10% (w/w) of trehalose to the juice formulation, also passing from an acidic to a basic media resulted in a considerable decreased in the *Lactobacillus salivarius* spp. *salivarius* viability. This is why, although the only addition of 10 % (w/w) of trehalose to the juice doubled the probiotic survival

to the gastric step, its survival to the whole gastrointestinal simulation was not significantly improved (p-value < 0.05).

As for the sublethal homogenization, its effect on the probiotic viability was dependent on both the addition of trehalose to the juice and the level of pressure applied. Regardless of the pressure applied, homogenization was observed to significantly increase the probiotic survival to both the gastric step and the whole digestion process (p-value < 0.05) in the juice containing no trehalose, but to significantly reduce those values in the juice containing 10% (w/w) of trehalose (p-value < 0.05). Regardless of the trehalose concentration, increasing from 50 to 100 MPa the homogenization pressure was reported to significantly reduce the percentage of cells remaining alive at the end of the gastric step. However, due to its different effect on the probiotic viability to the intestinal step, increasing from 50 to 100 MPa the homogenization pressure significantly reduced the probiotic survival to the whole gastrointestinal digestion in the juice containing no trehalose, but significantly increased that of the juice containing 10% (w/w) of trehalose. As a result of all this, the survival of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 to the *in vitro* digestion was maximum after homogenizing at 50 MPa the juice containing no trehalose, but reached a minimum value after homogenizing at 50 MPa the juice containing 10% (w/w) of trehalose.

In all cases, as it is evidenced in Fig. 3, the probiotic counts at the end of the digestion process resulted higher than 10^7 CFU/mL so the daily intake of 100 mL (less than half a glass) of any of these clementine juice-based liquids would provide the minimum dose required to provide a health benefit to the consumer (Collado et al., 2005; Sanders, 2008).

3.6. Effect of processing variables on anti-*Helicobacter pylori* effect of *Lactobacillus salivarius* spp. *salivarius*

Mean diameters of *Helicobacter pylori* growth inhibition by *Lactobacillus salivarius* spp. *salivarius* grown in different media are shown in Table 6. According to the results obtained from *in vitro* digestion assays, authors decided to only evaluate anti-*Helicobacter pylori* activity as affected by the addition of 10% (w/w) of trehalose to the juice before its inoculation and/or the juice homogenization at 50 MPa after incubation. Results showed that *Lactobacillus salivarius*

spp. *salivarius* strain CECT 4063 possesses the antagonistic activity to inhibit the growth of *Helicobacter pylori*, which was not significantly affected by the different growing media assessed. The validity of *Lactobacillus salivarius* as a probiotic to suppress *Helicobacter pylori* and thus reduce the inflammatory response was previously observed by Aiba et al. (1998). If as reported by Lin et al. (2011), anti-*Helicobacter pylori* activity is closely correlated with the concentration of organic acids and the pH value, those juices reaching a lower pH after the incubation with the probiotic (juices without trehalose) should exhibit a higher bactericidal activity against *Helicobacter pylori*. However, since clementine juice is naturally rich in flavonoids (hesperidin, narirutin, didymin, etc.) with antibacterial activity, differences in anti-*Helicobacter pylori* activity among probiotic beverages are minimal. In the particular case of MRS broth, the lack of flavonoids might be compensated with the higher microbial growth under optimal conditions.

4. Conclusions

Both physicochemical and microbial properties of clementine juice inoculated with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 were significantly affected by the addition of 10 or 20% of trehalose by weight to the juice formulation and/or the homogenization at 25, 50, 100 or 150 MPa. On one hand, increasing the trehalose concentration in the juice was observed to promote osmotic stress conditions and to significantly reduce the microbial counts, while notably increasing the probiotic resistance to both further homogenization and the shock produced by gastric juices during *in vitro* digestion. On the other hand, homogenization pressures below 150 MPa were reported to enhance (≤ 50 MPa) or maintain the amount of viable cells in the juice and to significantly increase the probiotic survival after both the gastric step and the whole gastrointestinal simulated process. Consequently, homogenizing at pressures not exceeding 100 MPa the juice containing no trehalose managed to significantly increase the amount of living cells after *in vitro* digestion with proven ability to inhibit the *Helicobacter pylori* growth.

Regarding antioxidant properties of the samples, both total phenols and flavonoids content, together with the antioxidant activity, were mainly improved by the growth of *Lactobacillus*

salivarius spp. *salivarius* CECT 4063 in the juice. Moreover, the expected increase in total phenols bioavailability due to the decrease in the average size of the particles after the homogenization step was only evident when 10% of trehalose by weight was added to the juice formulation. On the contrary, due to their lower average particle size, homogenized juices showed higher ability to be used as impregnating liquids. In summary, the two factors considered in the present study might be combined in an appropriate way in order to improve the health benefits provided by the consumption of clementine juice inoculated with *Lactobacillus salivarius* spp. *salivarus* CECT 4063.

Acknowledgements

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Table 1. Physicochemical properties of clementine juice at pH 6.5 non-inoculated (NI) and inoculated and incubated for 24 h at 37 ºC with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 as a function of trehalose concentration and/or the homogenization pressure.

%TREH	P (MPa)	a_w	pH	Brix	D[4,3]*	d_{90}**
	NI	0.9856 (0.0009)[fg]	6.567 (0.012)[i]	12.07 (0.06)[a]	227 (24)[de]	572 (72)[d]
0	**0**	0.9863 (0.0003)[g]	4.93 (0.06)[a]	12.0 (0.2)[a]	232 (8)[de]	577 (6)[d]
	25	0.9866 (0.0011)[g]	4.94 (0.06)[ab]	12.03 (0.10)[a]	41 (4)[a]	100 (6)[a]
	50	0.9862 (0.0012)[g]	4.94 (0.10)[ab]	12.0 (0.2)[a]	38 (5)[a]	93 (8)[a]
	100	0.9865 (0.0016)[g]	4.97 (0.12)[b]	11.97 (0.14)[a]	28.4 (0.8)[a]	79 (2)[a]
	150	0.9858 (0.0005)[fg]	4.96 (0.10)[ab]	11.77 (0.10)[a]	43 (3)[a]	101 (4)[a]
	NI	0.9803 (0.0008)[c]	6.770 (0.010)[j]	20.2 (0.4)[c]	238 (20)[e]	597 (54)[d]
10	**0**	0.9818 (0.0010)[cd]	5.45 (0.03)[c]	19.27 (0.15)[c]	211 (21)[bc]	530 (63)[bc]
	25	0.984 (0.002)[ef]	5.51 (0.06)[def]	18.60 (0.10)[b]	38.3 (1.1)[a]	107 (3)[a]
	50	0.9828 (0.0012)[de]	5.49 (0.03)[d]	18.60 (0.10)[b]	50 (3)[a]	90 (3)[a]
	100	0.9836 (0.0008)[e]	5.49 (0.17)[d]	18.87 (0.15)[b]	29 (3)[a]	75 (5)[a]
	150	0.9834 (0.0006)[de]	5.50 (0.02)[de]	18.90 (0.10)[b]	27.8 (0.8)[a]	69.2 (1.1)[a]
	NI	0.9726 (0.0010)[a]	6.803 (0.006)[k]	28.13 (0.06)[g]	206 (13)[b]	522 (31)[b]
20	**0**	0.9767 (0.0004)[b]	5.55 (0.04)[gh]	21.90 (0.10)[d]	221 (11)[cd]	563 (29)[cd]
	25	0.9782 (0.0003)[b]	5.53 (0.03)[fgh]	21.14 (0.10)[d]	34.3 (0.9)[a]	99 (2)[a]
	50	0.9777 (0.0003)[b]	5.54 (0.02)[ghi]	25.2 (1.1)[f]	31 (2)[a]	86 (3)[a]
	100	0.9773 (0.0010)[b]	5.55 (0.03)[gh]	24.70 (0.10)[e]	28 (3)[a]	76 (4)[a]
	150	0.9779 (0.0002)[b]	5.57 (0.02)[h]	24.93 (0.15)[ef]	27 (4)[a]	71 (8)[a]

Mean values and standard deviation in brackets.

* mean diameter over volume in microns.

** particle diameter at 90% in the cumulative distribution in microns.

[abc...] different superscripts in the same column indicate statistically significant differences (p < 0.05)

Table 2. Antioxidant properties of clementine juice at pH 6.5 non-inoculated (NI) and inoculated and incubated for 24 h at 37 ⁰C with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 as a function of trehalose concentration and/or the homogenization pressure.

%TREH	P (MPa)	vitamin C (mg AA/mL)	PHENOLS (mg GAE/mL)	FLAVONOIDS (mg QE/mL)	ABTS-TEAC (mg TE/mL)	DPPH (mg DPPH$_{red}$/mL)
0	**NI**	0.35 (0.05)^a	0.72 (0.03)^b	0.466 (0.014)^b	0.77 (0.07)^a	1.6 (0.6)^{bc}
	0	0.35 (0.02)^a	0.78 (0.07)^{cd}	1.01 (0.07)^g	1.14 (0.08)^d	1.52 (0.02)^{ab}
	50	0.33 (0.07)^a	0.754 (0.007)^{bc}	0.99 (0.03)^{fg}	0.98 (0.14)^c	1.5 (0.3)^{ab}
	100	0.35 (0.05)^a	0.724 (0.007)^b	0.954 (0.014)^{de}	0.95 (0.12)^{bc}	1.4 (0.3)^{ab}
10	**NI**	0.355 (0.005)^a	0.64 (0.05)^a	0.37 (0.08)^a	0.83 (0.12)^{ab}	1.87 (0.09)^d
	0	0.352 (0.008)^a	0.79 (0.08)^{cd}	0.904 (0.004)^c	1.00 (0.12)^c	1.7 (0.2)^{cd}
	50	0.34 (0.02)^a	0.85 (0.08)^e	0.97 (0.06)^{ef}	1.03 (0.06)^{cd}	1.39 (0.04)^a
	100	0.35 (0.02)^a	0.82 (0.08)^{de}	0.93 (0.03)^{cd}	0.8 (0.3)^a	1.39 (0.07)^a

Mean values and standard deviation in brackets.

[abc...] different superscripts in the same column indicate statistically significant differences ($p < 0.05$)

Table 3. Impregnation parameters of inoculated clementine juice as a function of trehalose concentration and/or the homogenization pressure.

%TREH	P (MPa)	γ_1	γ	X_1	X	ε_e
	0	0.091 (0.003)^c	0.012 (0.007)^b	0.150 (0.010)^d	0.19 (0,02)^{ab}	0.20 (0.02)^{ab}
0	50	0.03 (0.02)^b	0.006 (0.013)^b	0.052 (0.010)^b	0.23 (0,02)^b	0.24 (0.02)^{abc}
	100	-0.02 (0.02)^a	0.004 (0.007)^b	0.021 (0.010)^a	0.241 (0,002)^b	0.27 (0.04)^{bc}
	0	0.10 (0.03)^c	-0.015 (0.02)^{ab}	0.090 (0.010)^c	0.16 (0.03)^a	0.18 (0.05)^a
10	50	0.060 (0.010)^b	-0.06 (0.02)^{ab}	0.15 (0.02)^d	0.26 (0.02)^b	0.28 (0.05)^c
	100	0.049 (0.017)^b	-0.09 (0.04)^a	0.17 (0.02)^d	0.22 (0.07)^{ab}	0.29 (0.10)^c

Mean values and standard deviation in brackets.

[abc...] different superscripts in the same column indicate statistically significant differences ($p < 0.05$)

Table 4. Theoretical estimation of the amount of each active compound that is incorporated per gram of fresh apple (var. Granny Smith) during the vacuum impregnation with the different impregnation liquids.

%TREH	P (MPa)	10^{-8} CFU/g	vitamin C (mg AA/g)	PHENOLS (mg GAE/g)	FLAVONOIDS (mg EQ/g)	ABTS-TEAC (mg TE/g)	DPPH (mg $DPPH_{red}$/g)
0	**0**	1.3 (0.2)[d]	0.083 (0.010)[ab]	0.18 (0.02)[ab]	0.24 (0.03)[ab]	0.27 (0.03)[bc]	0.36 (0.04)[ab]
	50	1.14 (0.09)[c]	0.095 (0.007)[b]	0.22 (0.02)[bc]	0.29 (0.02)[b]	0.28 (0.02)[bc]	0.43 (0.03)[b]
	100	1.190 (0.009)[cd]	0.105 (0.008)[b]	0.217 (0.02)[abc]	0.286 (0.002)[b]	0.285 (0.002)[bc]	0.420 (0.003)[ab]
10	**0**	0.25 (0.04)[a]	0.069 (0.012)[a]	0.15 (0.03)[a]	0.18 (0.03)[a]	0.20 (0.03)[a]	0.33 (0.06)[a]
	50	0.71 (0.04)[b]	0.109 (0.007)[b]	0.27 (0.02)[c]	0.31 (0.02)[b]	0.33 (0.02)[c]	0.45 (0.03)[ab]
	100	0.28 (0.04)[a]	0.10 (0.03)[ab]	0.22 (0.08)[bc]	0.25 (0.09)[b]	0.22 (0.07)[ab]	0.38 (0.13)[ab]

Mean values and standard deviation in brackets.

[abc...] different superscripts in the same column indicate statistically significant differences ($p < 0.05$)

Table 5. *Lactobacillus salivarius* spp. *salivarius* CECT 4063 survival during the simulated gastrointestinal digestion as affected by the trehalose concentration and/or the homogenization pressure.

%TREH	P (MPa)	%VIAB$_{ST1}$	%VIAB$_{ST2}$	%VIAB$_{ST1+ST2}$
	0	23.3 (1.1)^a	121 (3)^e	28.1 (0.7)^c
0	**50**	39.85 (1.04)^d	122 (4)^e	50 (4)^e
	100	33.3 (1.2)^c	109 (3)^d	36.5 (0.9)^d
	0	51.3 (0.9)^e	59 (3)^b	30.2 (1.4)^c
10	**50**	29.1 (0.6)^b	37 (2)^a	10.6 (0.4)^a
	100	24.3 (0.4)^a	87 (3)^c	21.3 (0.7)^b

Mean values and standard deviation in brackets.

[abc...] different superscripts in the same column indicate statistically significant differences ($p < 0.05$)

Table 6. Inhibition of *Helicobacter pylori* by *Lactobacillus salivarius* spp. *salivarius* strain CECT 4063 as affected by the growing media.

%TREH	P (MPa)	Ø inhibition (mm)
0	0	17 (2)[a]
	50	17.7 (0.6)[a]
10	0	17 (2)[a]
	50	16.7 (0.6)[a]
MRS		16.7 (0.6)[a]
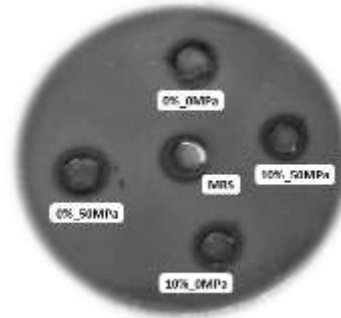
Mean values and standard deviation in brackets.

[abc...] different superscripts in the same column indicate statistically significant differences ($p < 0.05$)
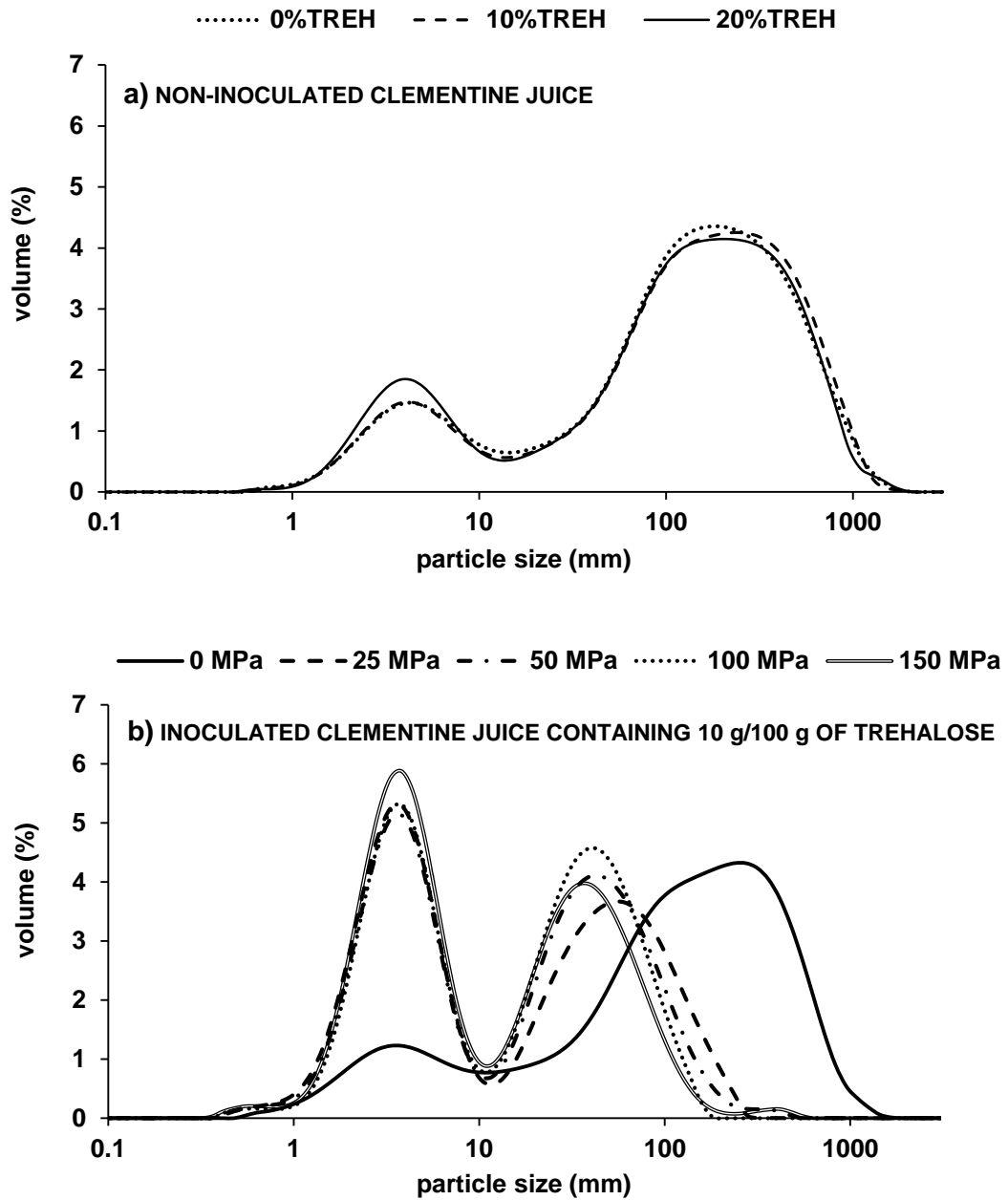
Fig. 1 Effect of the trehalose concentration (a) and the homogenization pressure (b) on clementine juice distribution curves for particle size.
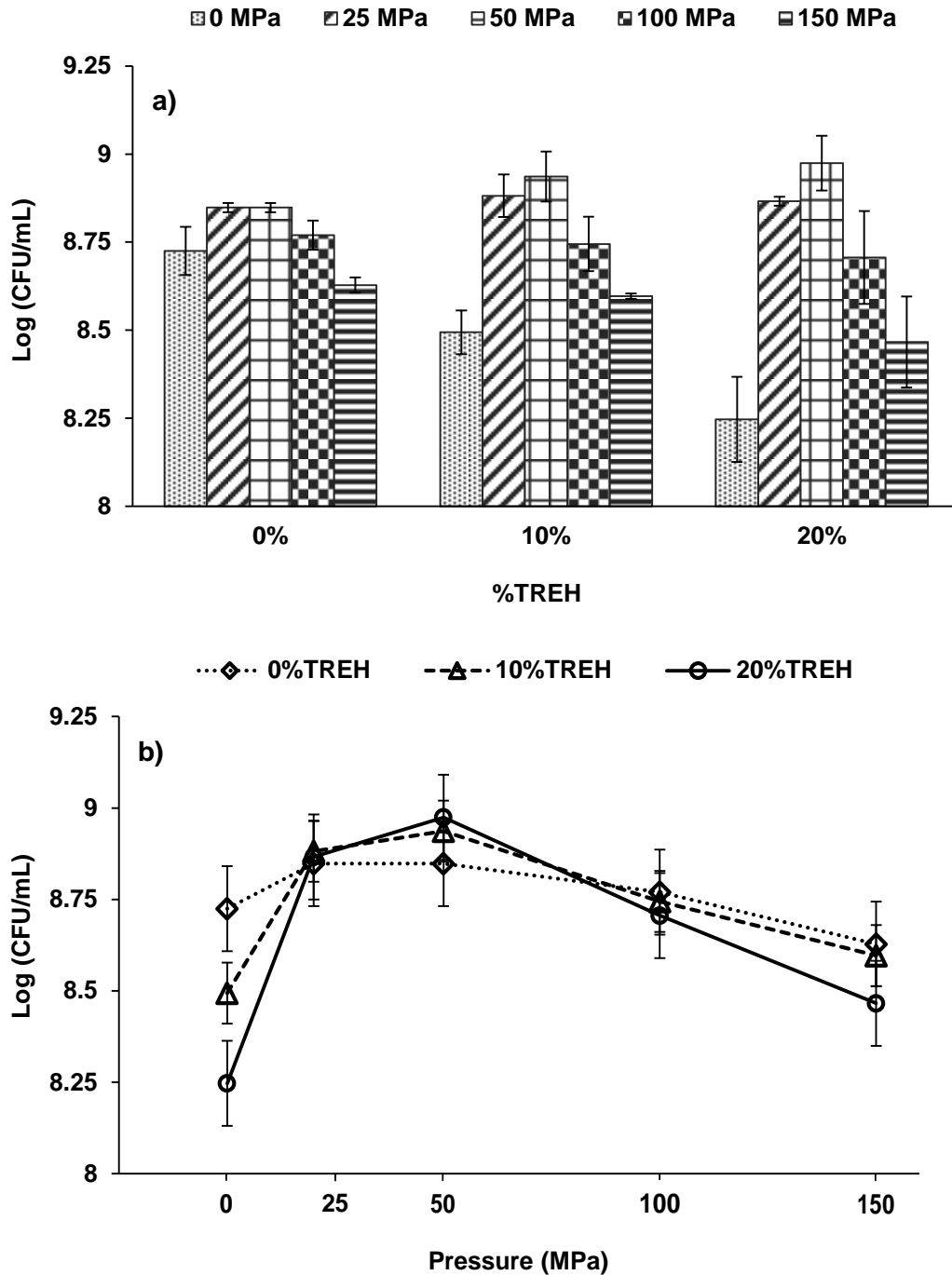
Fig. 2 Effect of trehalose concentration and/or the homogenization pressure on *Lactobacillus salivarius* spp. *salivarius* CECT 4063 growing: mean values and standard deviation as error bars (a) and graph of interactions and LSD intervals with a 95% confidence level (b).
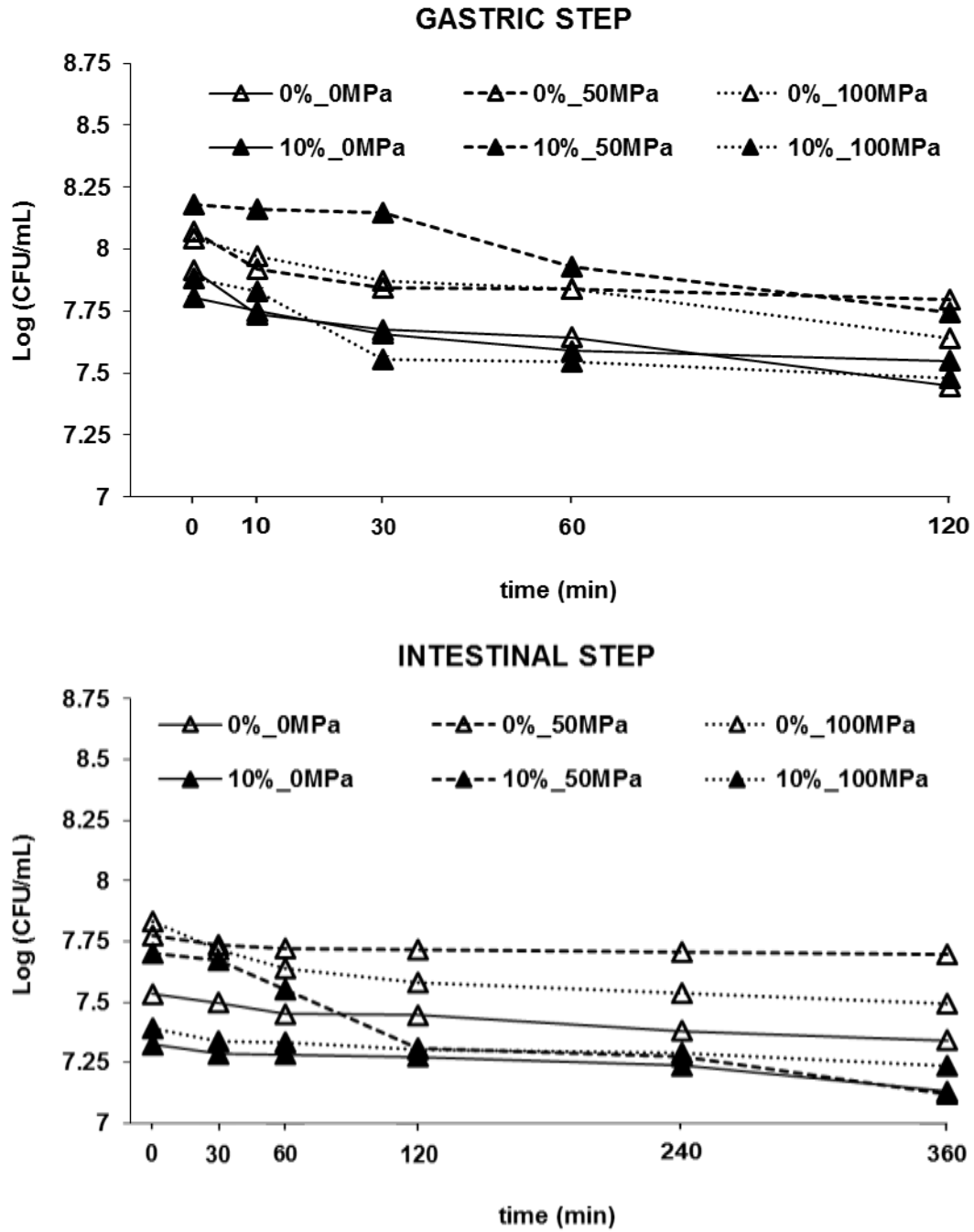
Fig. 3 Effect of trehalose concentration and/or the homogenization pressure on *Lactobacillus salivarius* spp. *salivarius* CECT 4063 survival to the *in vitro* gastrointestinal digestion.