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Fermentation of Clementine Juice with *Lactobacillus salivarius* spp. *salivarius* CECT 4063: Effect of Trehalose Addition and High-Pressure Homogenization on Antioxidant Properties, Mucin Adhesion, and Shelf Life

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Abstract: Fermentation of fruit juices with lactic acid bacteria enhances their antioxidant properties to a different extent depending on the microbial strain and the growing media composition, which can be modified by adding certain ingredients or applying a homogenization step. This study analyzed the effect of trehalose addition (10%, *w*/*w*) and homogenization at 100 MPa before or after *Lactobacillus salivarius* spp. *salivarius* CECT 4063 inoculation on the antioxidant profile and the microbiological properties of commercial clementine juice during 96 h fermentation. Antioxidant properties and viable cell count of 24 h-fermented juices during refrigerated storage (30 days at 4 °C) were also evaluated. Fermentation over 24 h reduced the microbial population and antioxidant content of clementine juice. Homogenizing the juice before inoculation enhanced the microbial growth but favored antioxidant degradation. Adding trehalose (10%, *w*/*w*) to the juice formulation and/or homogenizing at the fermented juice at 100 MPa for 24 h had a negative impact on viable counts and did not improve the microbial adhesion to intestinal mucosa. However, both techniques prevented antioxidant oxidation and cell decay during the storage of fermented juice under refrigeration, which should not last more than 15 days.

Keywords: fermentation; *Lactobacillus salivarius* spp. *salivarius* CECT 4063; clementine juice; mucin adhesion; antioxidant properties; trehalose; high-pressure homogenization

Citation: Burca-Busaga, C.G.; Betoret, N.; Seguí, L.; Barrera, C. Fermentation of Clementine Juice with *Lactobacillus salivarius* spp. *salivarius* CECT 4063: Effect of Trehalose Addition and High-Pressure Homogenization on Antioxidant Properties, Mucin Adhesion, and Shelf Life. *Fermentation* **2022**, *8*, 642. https://doi.org/10.3390/fermentation8110642

Academic Editor: Kurt A. Rosentrater

Received: 20 October 2022
Accepted: 11 November 2022
Published: 15 November 2022

1. Introduction

Consumers are increasingly aware of the benefits of a healthy lifestyle. This is evident in the growing demand for functional foods that promote the maintenance and improvement of health, such as probiotic foods [1]. Probiotics are defined as live microorganisms intended to provide health benefits when administered in adequate amounts [2]. For these microorganisms to be included in foods, they must be generally recognized as safe (GRAS) as well as fulfill other requirements, including resistance to acidic conditions of the stomach and the digestive enzymes of the tract, adherence to intestinal mucosa, competitive exclusion of pathogens, survival from industrial production and storage conditions, safety, and scientifically proven efficacy. Benefits derived from probiotic consumption include the prevention of diarrhea caused by pathogenic bacteria and viruses, the stimulation of the host's immune system, the reduction of the symptoms of lactose intolerance, and the prevention of constipation, among others [3,4]. In vitro and in vivo studies show that some lactic acid bacteria are also able to inhibit the growth of *Helicobacter pylori* and reduce the activity of the enzyme urease, required by the pathogen to resist the acidic conditions of the stomach [4]. This group includes several *Lactobacillus salivarius* strains, such as *L. salivarius* strains B37 and B60 [5], *L. salivarius* spp. *salicinius* AP-32 [6] and *L. salivarius* spp. *salivarius* CECT 4063 [7].

In addition to these benefits, biochemical changes taking place during fermentation with probiotics also contribute to enhance nutritional properties of certain foods. Fermentation of fruit juices with lactic acid bacteria has been shown to convert certain polyphenols into other metabolites with higher antioxidant activity and/or higher bioavailability [8]. In soy-derived products, fermentation with LAB improved protein and mineral bioavailability by reducing antinutritional factors, such as phytic acid [9].

As regards the viability of probiotics in food products, it depends on several factors [10]: (a) factors related to the composition of the food matrix, such as pH, salt and sugar content or oxygen concentration; (b) factors related to the microorganism, such as the specific strain or the inoculum concentration; and (c) factors related to the processing and storage conditions. In the manufacture of a probiotic snack by means of vacuum impregnation and further dehydration [11], the higher the microbial concentration in the impregnation liquid, the greater the counts in the vacuum-impregnated product. Then, the decrease in the microbial population undergone by the impregnated product during its further processing can be reduced in different ways. Techniques employed to extend the shelf life of probiotics in foods from production to consumption include the modification of processing and storage conditions, the addition of different protective agents, or encapsulation [10]. Among protective agents, trehalose is a nonreducing disaccharide made up of two glucose molecules that acts as a reserve sugar and confers protection against abiotic stress in various organisms [12]. In dehydration processes, trehalose favors the fluid state of lipids, thus preventing their fusion, phase separation and the rupture of biological membranes. In addition, the liquid-to-gel transition is delayed by replacing water molecules with trehalose molecules [13].

The functionality of probiotics can also be enhanced by means of high-pressure homogenization (HPH). This is a nonthermal technology that is usually applied to liquid foods to inactivate enzymes, decrease the microbial load, improve their sensory and technological quality, and increase the availability of active compounds [14]. On probiotic microorganisms, HPH at sublethal pressures has been proven to be effective in improving hydrophobicity, self-aggregation and resistance to biological stress in different food matrices, in addition to preserving their viability during storage in refrigeration [15,16]. Cellular hydrophobicity is directly related to the adhesion capacity of the microbial strain and, as previously mentioned, its interaction capacity with the gut. Resistance to harsh conditions induced by HPH includes the probiotic passage through the gastrointestinal tract [7].

The objective of the present study was to evaluate the effect of trehalose addition (10% *w*/*w*) and homogenization (100 MPa) before or after *Lactobacillus salivarius* spp. *salivarius* CECT 4063 inoculation on the microbial growth and antioxidant profile of commercial clementine juice during 96 h fermentation. Mucin adhesion, antioxidant properties and viable cell counts of 24 h-fermented juices during refrigerated storage (30 days 4 °C) were also evaluated. The purpose was to obtain a liquid with maximum microbial and antioxidant contents to be used as an impregnating liquid in vacuum-impregnation processes.

2. Materials and Methods

2.1. Raw Materials and Microbial Strain

Pasteurized and refrigerated squeezed clementine juice was purchased from a local supermarket (Valencia, Spain) and kept refrigerated at 4 °C in its own airtight Tetra Brik® container until use. Food-grade trehalose from tapioca starch (TREHA™) was supplied by Cargill Inc. (Barcelona, Spain).

Lactobacillus salivarius spp. *salivarius* CECT 4063 from the Spanish Type Culture Collection (University of Valencia, Burjassot, Spain) was used in this study. The strain was cultured in MRS broth (Scharlau Chemie®, Barcelona, Spain) at 37 °C. After 24 h in aerobiosis, broths with a microbial concentration of 10^9 cfu/mL measured by plate count were obtained.

2.2. Juice Conditioning, Fermentation, and Storage

As described by Barrera et al. [7], 5 g/L of yeast extract (Panreac Química S.L.U., Barcelona, Spain) were added to commercial clementine juice together with 9.8 g/L of food-grade sodium bicarbonate so that its pH was raised from 3.8 to 6. In samples containing trehalose, 10% (*w*/*w*) of such disaccharide was incorporated into the juice formulation. Once the ingredients were dissolved, part of the liquid was homogenized at 100 MPa in a laboratory-scale high-pressure homogenizer (Panda Plus 2000, GEA-Niro Soavi, Parma, Italy). Then, both homogenized (HPH) and unhomogenized (non-HPH) juices were inoculated with 4 mL/L of MRS broth containing the activated microorganism and incubated at 37 °C in aerobiosis for 96 h. After 24 h of incubation, part of the unhomogenized samples was homogenized at 100 MPa and returned to incubation to complete the 96 h of fermentation (I+HPH). Fermented samples were collected at 0, 24 (before and after homogenizing), 48, 72, and 96 h for microbiological and chemical analysis. Fermenting for such long periods will not only provide information about the effect of the processing variables on the microbial growth during the log phase but also on the exponential decrease in the number of living cells during the death phase. Non-HPH and I+HPH liquids (with and without 10% (*w*/*w*) of trehalose) that were fermented for 24 h underwent the storage study at 4 °C for 30 days. Sampling in this case took place at days 0, 1, 2, 3, 7, 10, 15, 21, and 30.

2.3. Microbial Counts

Colony counts of live *Lactobacillus salivarius* spp. *salivarius* CECT 4063 present in each juice were conducted throughout fermentation and storage by serial dilution in sterile peptone water and plating on the surface of MRS agar (Scharlau Chemie®, Barcelona, Spain) plates. Then, the plates were incubated at 37 °C for 24 h under aerobic conditions.

2.4. pH and Brix

Brix values were obtained from the refractive index of the juices measured at 20 °C in an optical refractometer (Abbe NAR-T3, ATAGO™, Tokyo, Japan). The pH of the different liquids was measured at 25 °C with a benchtop pH meter (S20 SevenCompact Easy™, Mettler Toledo, Barcelona, Spain) correctly calibrated with buffer solutions at pH 4.0 and 7.0.

2.5. Antioxidant Properties

2.5.1. Total Phenolic Content (TPC)

Total phenolic content was determined by the Folin–Ciocalteu method [17], based on the ability of phenolic compounds to reduce a mixture of phosphotungstic and phosphomolybdic acids in a basic medium to form blue tungsten and molybdenum oxides. To carry out this analysis, 125 µL of the sample diluted in bidistilled water in a 1:5 (*v*/*v*) ratio was mixed with 125 µL of the Folin–Ciocalteu reagent and 500 µL of bidistilled water. After 6 min in darkness, 1.25 mL of sodium bicarbonate at 7.5% (*w*/*v*) and 1 mL of bidistilled water were added. A blank in which the sample was replaced with distilled water was used as a reference. After 90 min in darkness, the absorbance was measured at 760 nm in a Helios Zeta UV/vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Results were expressed in mg of gallic acid equivalents per mL of juice (mg GAE/mL).

2.5.2. Total Flavonoid Content (TFC)

The total flavonoid content (TFC) was determined by the colorimetric method of aluminum chloride described by Luximon-Ramma et al. [18]. Briefly, 1.5 mL of the sample diluted in bidistilled water in a 1:5 (*v*/*v*) ratio was mixed with 1.5 mL of aluminum chloride in methanol at 2% (*w*/*v*). A blank in which the sample was replaced with distilled water was used as a reference. After 10 min in darkness, the absorbance was measured at 368 nm in a Helios Zeta UV/vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Results were expressed in mg of quercetin equivalents per mL of juice (mg QE/mL).

2.5.3. Antioxidant (AO) Activity

Total antioxidant capacity of juice samples was measured by both ABTS-TEAC and DPPH assays. The ABTS-TEAC assay is based on measuring the discoloration undergone by the radical 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) when reduced in the presence of oxygen-donor antioxidants [19]. For the ABTS-TEAC assay, 90 µL of each sample diluted in bidistilled water in a 1:5 (v/v) ratio was added into 2910 µL of a 7 mM $ABTS^+$ solution in phosphate buffer (A_{734} nm = 0.7 ± 0.01). A white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. After 6 min of reaction in the dark, the absorbance was measured at 734 nm in a Helios Zeta UV/vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Results were expressed in mg of Trolox equivalents per mL of juice (mg TE/mL).

DPPH assay is based on measuring the discoloration undergone by the radical 2,2-diphenyl-1-picrylhydrazyl due to the donation of hydrogen atoms in the presence of antioxidant compounds [20]. To carry out this analysis, 100 µL of the sample diluted in bidistilled water in a 1:5 (v/v) ratio was added into 2000 µL of a 100 mM solution of $DPPH^\bullet$ in methanol and 900 µL of methanol (purity ≥ 99%). A white reference sample was prepared by replacing the volume of sample by the same volume of bidistilled water. After 30 min of reaction in the dark, the absorbance was measured at 517 nm in a Helios UV/vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Results were expressed in mg of Trolox equivalents per mL of juice (mg TE/mL).

2.5.4. Quantification of Individual Flavonoids by HPLC

Extraction and analysis of the major flavonoids, i.e., narirutin (NAR), hesperidin (HESP) and didymin (DID), was adapted from Cano et al. [21]. Aliquots of 6 mL of juice were mixed with 4 mL of a 1:1 (v/v) solution of dimethyl sulfoxide in methanol. After stirring for 1 min at 1000 rpm in a GFL compact orbital shaker 3005, the mixture was centrifuged at 4 °C for 30 min at 10,000 rpm (Medifriger BL-S, J.P. Selecta, Barcelona, Spain). The collected supernatant was filtered through a 0.45 µm nylon filter and directly injected into a C18 reversed-phase column (250 × 4.6 mm and 5 µm) installed in a Waters Alliance 2695 HPLC (Waters Inc., Milford, CT, USA) equipped with a 996 photo diode array detector. A mobile phase consisting of acetonitrile (solvent A) and 0.6% (v/v) acetic acid (solvent B) was used for analysis. The test conditions were as follows: 10% of solvent A for 2 min, increase the concentration of solvent A progressively until reaching 50% during the following 18 min, hold for 5 min, and return to the initial conditions in 5 min. A constant flow rate of 1 mL/min was used. Compounds were identified comparing their retention times (13.7 min, 14.3 min and 17.2 min for NAR, HESP and DID, respectively) and UV-vis spectra at 280 nm with the corresponding standards. Concentrations were obtained using an external standard curve.

2.6. Adhesion Assay

Based on the method described by Izquierdo et al. [22], *Lactobacillus salivarius* spp. *salivarius* cells from 24-hour-old cultures grown in MRS broth or conditioned clementine juice were collected by centrifugation (4000 rpm for 10 min at 4 °C), washed twice, and resuspended in phosphate-buffered saline (PBS) solution. Next, the cell suspension absorbance measured at 600 nm was adjusted to 0.25 ± 0.05, equivalent to a microbial concentration of 10^7–10^8 cfu/mL. Then, cell suspensions were labeled in the dark with 75 µm carboxyfluorescein diacetate (Sigma Aldrich, Steinheim, Germany) for 1 h at room temperature. Finally, microbial cells were washed twice and resuspended in PBS.

In parallel, 500 ppm solutions in PBS were prepared for each of the three different proteins assayed: mucin from porcine stomach (MUC), collagen from calf skin (COL) and bovine serum albumin (BSA), all purchased from Sigma-Aldrich (Steinheim, Germany). Then, 100 µL of each solution was loaded into black polystyrene 96-well cell culture plates (Thermo Fisher Scientific, Nunc Edge 2.0, Waltham, MA, USA) and incubated overnight at 4 °C. To remove unadhered proteins, the wells were washed three times with 200 µL

PBS. Next, 100 µL of working labelled bacterial suspension was added per well. After incubating the plates for 1 h at 37 °C, the wells were washed twice with 200 µL PBS to remove unbound bacteria and 100 µL of PBS were added to each well containing adhered bacteria. The released fluorescence was red in a FluoroskanTM microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 485 nm excitation and 538 nm emission. Adhesion was expressed as the percentage of fluorescence recovered after binding relative to the fluorescence of the bacterial suspension initially added to the wells.

2.7. Statistical Analysis

The statistical significance of the results was evaluated with the Statgraphics Centurion XVI tool, by means of simple and multivariate analysis of variance with a 95% confidence level.

3. Results and Discussion

3.1. Fermentation of Clementine Juice by Lactobacillus salivarius spp. salivarius CECT 4063 as Affected by Trehalose Addition and/or Juice High-Pressure Homogenization

Figure 1 shows the effect of the homogenization treatment applied and the addition of trehalose (10%, *w*/*w*) on the *Lactobacillus salivarius* spp. *salivarius* CECT 4063 content of conditioned clementine juice throughout the fermentation process. As can be observed, the variation in cell counts over the incubation time resembled that of the theoretical variation showing the microbial growth under limited conditions of volume and nutrient content. In all cases, maximum growth occurred after 24 h of fermentation, which meant a slight decrease in Brix (from 19.3 ± 0.2 to 18.6 ± 0.4 and from 12.8 ± 0.5 to 12.4 ± 0.6 for juices formulated with and without trehalose, respectively) and a notable decrease in pH (from 6.54 ± 0.12 to 4.368 ± 0.012 for juices formulated with and without trehalose) due to lactic acid production. This led to the cell death phase, in which both Brix and pH remained almost constant.

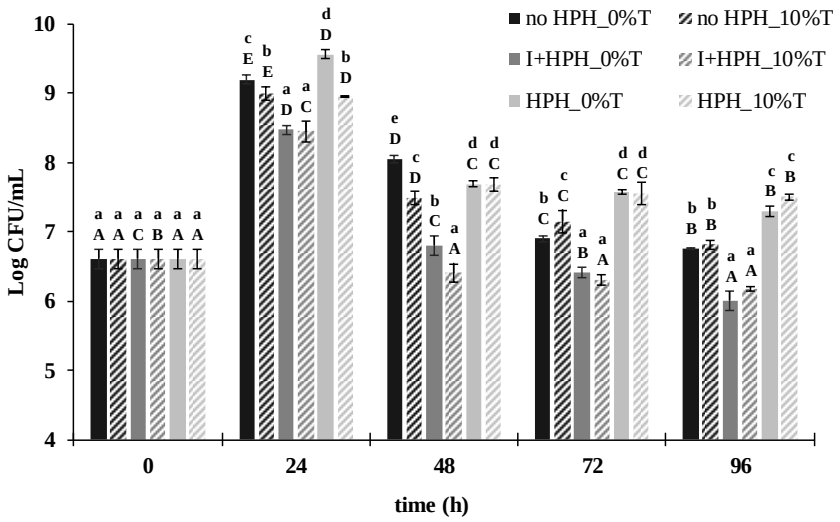

Figure 1. Microbial counts in clementine juice subjected to different treatments along their fermentation with *L. salivarius* spp. *salivarius* CECT 4063 for 4 days. Error bars represent the standard deviation of 4 replications. $^{a–e}$ In the same time and $^{A–D}$ in the same series indicate statistically significant difference with a 95% confidence level ($p < 0.05$).

As shown in Table 1, the increase in the microbial population was significantly enhanced ($p < 0.05$) by the homogenization of the juice at 100 MPa before its inoculation (treatment HPH_0%T). It follows from this that considerable reduction in the pulp particle size reported for HPH treatments applied to citrus juices as a result of pectin depolymerization might not only increase the juice cloudiness and reduce the juice viscosity [23,24] but also notably increase precocious availability of some chemical components that are

essential for the lactobacillus strain growth. In milk for cheesemaking, as an example, significant increase in the availability of low-molecular-weight peptides and free fatty acids such as oleic acid was observed by Burns et al. [25] after homogenization at 100 MPa. In orange juice, decrement of the size of the cloud fraction after homogenization at 200 and 300 MPa significantly improved the extractability of flavonoids and increased the amount of soluble flavanones [26]. Conversely, as a preservation technique, the homogenization of the juice that had been previously inoculated and incubated for 24 h at 37 °C caused a significant microbial inactivation, as deduced from the lowest log increase values reached by the I+HPH juices in this interval. In the same way, cell exposure to osmotic stress resulting from trehalose addition to the growing media and the subsequent Brix increase from 12.8 ± 0.4 to 19.3 ± 0.4 had a negative impact on the growth and multiplication of bacteria. In any case, the microbial content of the juices after 24 h of fermentation ranged between (2.9 ± 0.4) × 10^8 and (3.69 ± 0.6) × 10^9 cfu/mL, which is higher than the minimum concentration of 10^6–10^7 cfu/mL required to make an EU-based health claim [27].

Table 1. Log increase (values > 0) or log decrease (values < 0), respectively, showing the *L. salivarius* spp. *salivarius* CECT 4063 growth or demise from the juices along their fermentation.

Treatment		Interval			
		0–24 h	**24–48 h**	**24–72 h**	**24–96 h**
non-HPH	0%T	2.54 ± 0.06 ^{dD}	−1.13 ± 0.05 ^{dC}	−2.26 ± 0.05 ^{aB}	−2.400 ± 0.014 ^{abA}
	10%T	2.33 ± 0.10 ^{cD}	−1.48 ± 0.10 ^{cC}	−1.81 ± 0.16 ^{bB}	−2.13 ± 0.06 ^{cA}
I+HPH	0%T	1.70 ± 0.07 ^{aD}	−1.70 ± 0.14 ^{bC}	−2.11 ± 0.08 ^{aB}	−2.51 ± 0.14 ^{aA}
	10%T	1.68 ± 0.15 ^{aC}	−2.08 ± 0.13 ^{aB}	−2.18 ± 0.07 ^{aAB}	−2.32 ± 0.03 ^{bA}
HPH	0%T	2.68 ± 0.07 ^{eC}	−1.70 ± 0.04 ^{bB}	−1.80 ± 0.03 ^{bB}	−2.05 ± 0.08 ^{cA}
	10%T	2.153 ± 0.010 ^{bC}	−1.16 ± 0.09 ^{dB}	−1.3 ± 0.2 ^{cB}	−1.52 ± 0.04 ^{dA}

$^{a–e}$ In the same column and $^{A–D}$ in the same row indicate statistically significant difference with a 95% confidence level ($p < 0.05$). Mean value of 4 replications ± standard deviation.

As regards the decrease in the microbial population observed after 24 h of fermentation, it was initially very abrupt for treatments HPH and I+HPH (interval 24–48 h), but in the end (interval 24–96 h) it was significantly less evident for treatment HPH. This suggests that *Lactobacillus salivarius* spp. *salivarius* CECT 4063 survival in adverse conditions, basically due to nutrient depletion, low pH and the accumulation of organic acids with antimicrobial activity resulting from cellular metabolism, was not in this case enhanced by its sublethal homogenization. Instead, the homogenization of the juice prior to inoculation seemed to reinforce the ability of some food components to protect cells from acidic stress and/or that of antioxidant compounds to prevent oxidative stress [28]. In addition, the addition of 10% (*w*/*w*) of trehalose to the juice prevented in all cases the lactobacillus strain from decay after 24 h of fermentation, just as previously observed during the freeze-drying of vacuum-impregnated apple slices and after 30-day storage of apple snacks that had been dried with air at 40 °C for 48 h [29] or at the end of the gastric step in the course of in vitro digestion of clementine juice inoculated with *Lactobacillus salivarius* CECT 4063 [7]. Consequently, the minimal microbial count (1.0 ± 0.3) × 10^6 cfu/mL) was achieved after fermenting for 96 h the clementine juice that was first inoculated and incubated for 24 h at 37 °C and then homogenized at 100 MPa.

As deduced from the results obtained at time 0 h (Table 2), homogenizing the juice at 100 MPa before its inoculation affected neither the TPC nor the ability to scavenge DPPH$^{\bullet}$ free radicals, but significantly increased and decreased the TFC and the ability to scavenge $ABTS^{+}$ free radicals, respectively. Regarding trehalose addition, it hardly affected the AO activity measured by the application of both DPPH$^{\bullet}$ and $ABTS^{+}$ free radicals, but significantly increased the TPC and TFC of unhomogenized juice samples. These results confirm the ability of such disaccharides to prevent the oxidation of certain phenolic compounds reported by Kopjar et al. [30]. However, by homogenizing the juice

and reducing the size of the pulp, certain antioxidants (such as ascorbic acid or vitamin C) might be more exposed to light and dissolved oxygen [31] and the amount of trehalose added to the juice formulation is not enough to prevent their rapid degradation. Probably for this reason, the AO activity measured by the ABTS method had significantly lower values in HPH juices. When analyzed by the DPPH$^{\bullet}$ free radical method, the AO activity of the juices was much lower and was not affected by the process variables, which may be due to the lower affinity of this radical for hydrophilic compounds [32].

Table 2. Antioxidant properties of clementine juice subjected to different treatments along their fermentation with *L. salivarius* spp. *salivarius* CECT 4063 for 4 days.

		t (h)	Total Phenols (mg GAE/mL)	Total Flavonoids (mg QE/mL)	AO_DPPH (mg TE/mL)	AO_ABTS (mg TE/mL)
no HPH	0%T	0	0.84 ± 0.08^{aAB}	0.747 ± 0.014^{aA}	0.33 ± 0.02^{aA}	1.35 ± 0.06^{dD}
		24	1.81 ± 0.13^{dC}	2.09 ± 0.03^{bcD}	0.51 ± 0.04^{cB}	1.61 ± 0.08^{cE}
		48	0.99 ± 0.09^{bB}	1.65 ± 0.07^{cC}	0.31 ± 0.04^{abA}	0.92 ± 0.05^{bC}
		72	0.86 ± 0.06^{abAB}	1.64 ± 0.07^{cC}	0.32 ± 0.03^{cA}	0.85 ± 0.04^{bB}
		96	0.75 ± 0.09^{aA}	1.130 ± 0.002^{bB}	0.309 ± 0.012^{cA}	0.69 ± 0.07^{bA}
	10%T	0	0.90 ± 0.05^{bB}	0.89 ± 0.04^{bA}	0.31 ± 0.03^{aAB}	1.34 ± 0.09^{cdC}
		24	2.00 ± 0.10^{eC}	2.19 ± 0.11^{cD}	0.49 ± 0.02^{bcC}	1.53 ± 0.02^{cD}
		48	0.79 ± 0.03^{aAB}	1.4 ± 0.2^{aC}	0.34 ± 0.05^{bB}	0.78 ± 0.03^{aB}
		72	0.78 ± 0.03^{aA}	1.15 ± 0.06^{aB}	0.310 ± 0.011^{cAB}	0.719 ± 0.014^{aA}
		96	0.7 ± 0.2^{aA}	1.10 ± 0.03^{aB}	0.28 ± 0.03^{bA}	0.678 ± 0.005^{abA}
I + HPH	0%T	0	0.81 ± 0.04^{aA}	0.747 ± 0.014^{aA}	0.33 ± 0.02^{aD}	1.26 ± 0.04^{bcC}
		24	1.5 ± 0.2^{cD}	1.99 ± 0.09^{bC}	0.37 ± 0.03^{aE}	1.35 ± 0.06^{bD}
		48	1.37 ± 0.13^{cCD}	2.00 ± 0.05^{dC}	0.28 ± 0.03^{aC}	1.23 ± 0.05^{dC}
		72	1.26 ± 0.04^{cBC}	1.87 ± 0.14^{dBC}	0.18 ± 0.02^{aB}	1.11 ± 0.07^{cB}
		96	1.18 ± 0.05^{cB}	1.8 ± 0.2^{dB}	0.116 ± 0.006^{aA}	0.76 ± 0.07^{bA}
	10%T	0	0.90 ± 0.05^{bA}	0.89 ± 0.04^{bA}	0.31 ± 0.03^{aC}	1.19 ± 0.08^{bB}
		24	1.03 ± 0.14^{bB}	1.65 ± 0.02^{aD}	0.46 ± 0.03^{bD}	1.38 ± 0.13^{bC}
		48	0.97 ± 0.07^{bAB}	1.56 ± 0.06^{bcC}	0.29 ± 0.02^{aC}	1.14 ± 0.02^{cB}
		72	0.96 ± 0.09^{bAB}	1.55 ± 0.03^{bBC}	0.214 ± 0.008^{bB}	1.1 ± 0.2^{cB}
		96	0.94 ± 0.05^{bAB}	1.50 ± 0.05^{cB}	0.119 ± 0.003^{aA}	0.69 ± 0.03^{bA}
HPH	0%T	0	0.84 ± 0.04^{aA}	0.99 ± 0.02^{dA}	0.30 ± 0.01^{aA}	0.83 ± 0.04^{aC}
		24	0.83 ± 0.03^{aA}	1.58 ± 0.10^{aC}	0.51 ± 0.07^{cD}	0.88 ± 0.03^{aC}
		48	0.80 ± 0.06^{aA}	1.44 ± 0.12^{abB}	0.47 ± 0.03^{cCD}	0.82 ± 0.11^{aC}
		72	0.81 ± 0.06^{aA}	1.44 ± 0.09^{bB}	0.41 ± 0.03^{dBC}	0.69 ± 0.08^{aB}
		96	0.75 ± 0.07^{aA}	1.06 ± 0.06^{aA}	0.39 ± 0.06^{dB}	0.59 ± 0.10^{aA}
	10%T	0	0.80 ± 0.04^{aC}	0.94 ± 0.04^{cA}	0.33 ± 0.03^{aA}	0.80 ± 0.06^{aB}
		24	0.82 ± 0.05^{aC}	1.6 ± 0.2^{aC}	0.50 ± 0.02^{cD}	0.88 ± 0.05^{aC}
		48	0.79 ± 0.08^{aBC}	1.53 ± 0.14^{bcC}	0.47 ± 0.04^{cC}	0.79 ± 0.03^{aAB}
		72	0.72 ± 0.05^{aA}	1.45 ± 0.06^{bC}	0.426 ± 0.014^{dB}	0.73 ± 0.07^{aAB}
		96	0.72 ± 0.05^{aAB}	1.20 ± 0.04^{aB}	0.359 ± 0.013^{dA}	0.72 ± 0.08^{bA}

For each column, $^{a–e}$ in the same time and $^{A–E}$ in the same treatment indicate statistically significant difference with a 95% confidence level ($p < 0.05$). Mean ± standard deviation of 4 replications.

All the AO properties analyzed improved within the first 24 h of fermentation and then progressively decreased (Table 2). Increase in TPC was previously observed in heat- and HHP-treated lychee juice after fermentation for 18 h by *Lactobacillus casei* at 30 °C due to large polymeric phenolics degradation by the microbial strain [31]. In addition, in blueberry juice, the TPC increased by 6.1–81.2% and the AO capacity in vitro was enhanced by at least 34% under fermentation at 37 °C for 48 h with autochthonous lactic acid bacteria due to derivation of phenolics among themselves, the metabolism of organic acids and other metabolic pathways [33]. In the present study, increase in TPC and TFC

after fermentation of clementine juice for 24 h with *Lactobacillus salivarius* CECT 4063 ranged between 3.1 ± 0.7% and 123 ± 12% and between 56 ± 4% and 180 ± 4%, respectively.

Increase in TPC and TFC was minimum for HPH juices and maximum for non-HPH ones, which also underwent the highest microbial growth in the same interval. Small concentrations of dissolved oxygen in juices summited to high pressure treatments were reported in previous studies to accelerate the oxidation of ascorbic acid [31] and to cause the degradation of several nutraceutical compounds, including anthocyanins and polyphenols, over time [34]. Contrary to what has been previously commented for unfermented samples, the homogenization at 100 MPa after lactic acid fermentation for 24 h reduced both TPC and TFC of the juice, especially when trehalose was added to its formulation. From this, it follows that the phenolic compounds resulting from the microbial activity were more sensitive to high homogenization pressures than those naturally present in the juice. Differences found in TPC and TFC increase depending on the treatment did not significantly affect the rise in the capacity to scavenge free radicals of clementine juice due to fermentation, which was calculated in percentage as the difference between the values obtained for 24 h-fermented and unfermented liquids referred to the values obtained for unfermented liquids and were around 57 ± 8% and 11 ± 4% for $DPPH^{\bullet}$ and $ABTS^{+}$, respectively. Only the absence of trehalose seemed to slow down the increase in the AO activity measured by the $DPPH^{\bullet}$ free radical method in sample I+HPH, as deduced from the values obtained for unfermented and 24 h-fermented liquids, shown in Table 2.

To confirm these results obtained by spectrophotometric analysis techniques, the content of some of the most abundant flavonoids present in clementine juice were analyzed by chromatographic techniques. Figure 2 shows the concentration of hesperidin, narirutin, and didymin in each of the juices analyzed before and after 24 h of fermentation at 37 °C with *Lactobacillus salivarius* spp. *salivarius* CECT 4063. Among the flavonoids analyzed, hesperidin was found to be the most abundant in clementine juice, followed by narirutin and didymin. Values obtained were similar to those reported by Cano et al. [21] in a study on clementine pulp of different varieties, with a hesperidin content between 132 and 354 mg/kg and a narirutin content between 26 and 76 mg/kg. In a different study on fresh ortanique juice [23], hesperidin, narirutin and didymin contents were 116 ± 3 mg/L, 41.75 ± 0.02 mg/L and 14.3 ± 0.3 mg/L, respectively, and these values were not affected by the homogenization in the range between 5 and 30 MPa.

Focusing on freshly inoculated samples, only hesperidin was significantly ($p < 0.05$) more abundant in the juices that were homogenized at 100 MPa before its inoculation with the microbial strain (HPH samples). These samples were the ones that also presented the lowest AO activity measured by the $ABTS^{+}$ free radical method. After fermentation for 24 h, the expected increase in each individual flavonoid content was only evident in those juices that were subsequently homogenized at 100 MPa (I+HPH samples). However, according to the spectrophotometric analysis technique, non-HPH juices were the ones with the highest content of total flavonoids after 24 h of fermentation. Possibly, the fact of using quercetin with maximum absorbance at 368 nm as a reference flavonoid to express the total flavonoid content is the cause of such discrepancy between the results obtained by the different analysis techniques used.

Oxidation reactions continued 24 h after the start of fermentation, which—together with the loss of viability of the lactobacillus strain—caused a decline in all the AO properties of the juice. Degradation of AO compounds was notably less evident for HPH samples, in which the microbial decay was also observed to be minimum. On the contrary, the decrease in TPC, TFC and in the ability to scavenge the $ABTS^{+}$ free radical (calculated in percentage, as the difference between the values obtained for 96 h-fermented and 24 h-fermented liquids refers to the values obtained for 24 h-fermented liquids shown in Table 2) was maximum in non-HPH_0%T juice (59 ± 5%, 35 ± 12% and 43 ± 3%, respectively) and more or less slightly increased when adding 10% (*w*/*w*) of trehalose to its composition (66 ± 9%, 49.7 ± 1.4% and 55.8 ± 0.3%, respectively). In a different study, trehalose addition to orange jelly reduced from 27.8% to 9.14% the loss of phenols during storage [35]. In the present

study, positive influence of trehalose on the phenol content was also observed for I+HPH samples. As a result of these changes in the content of antioxidant compounds, the ability to scavenge the $ABTS^{+}$ free radical after fermenting the juices for 96 h with *Lactobacillus salivarius* CECT 4063 was even lower than that of unfermented samples.

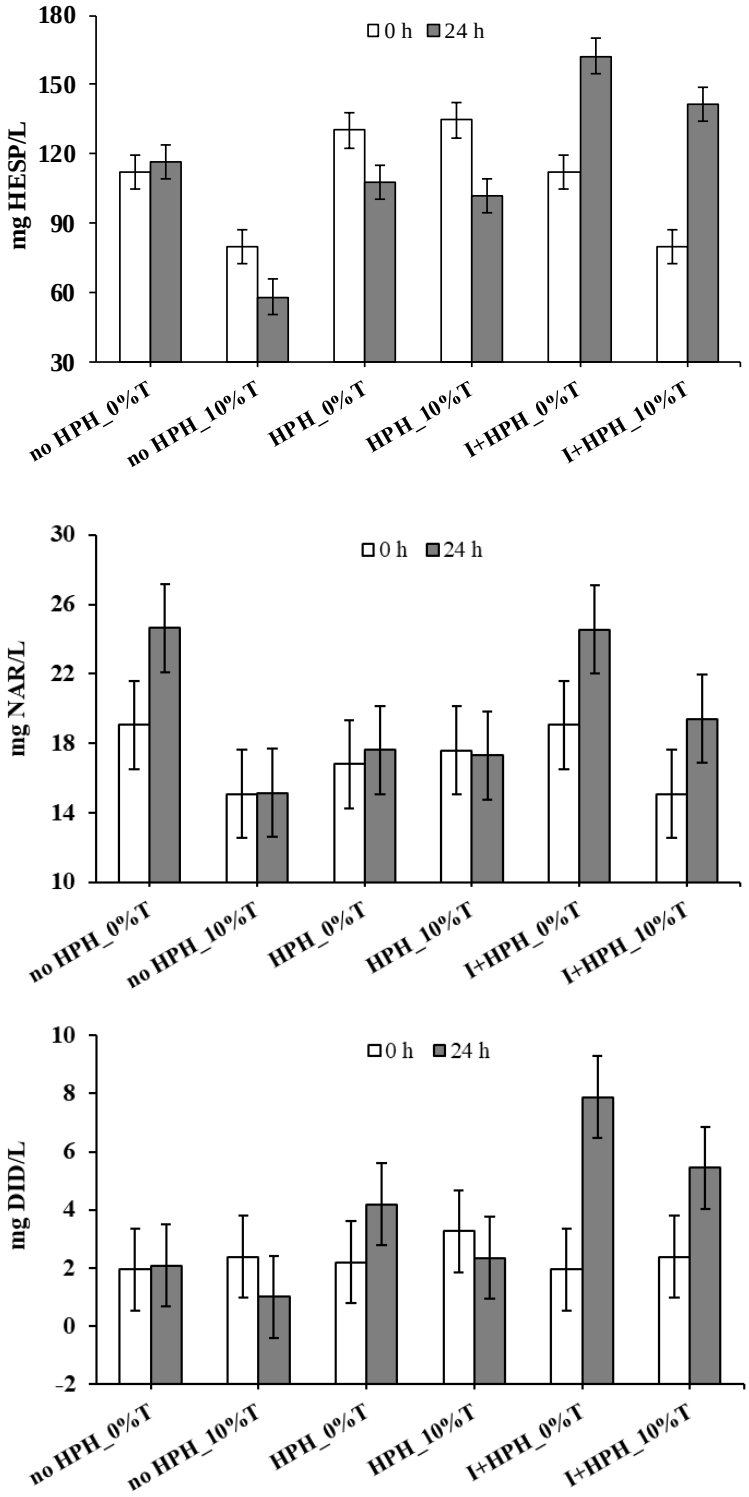

Figure 2. Hesperidin (HESP), narirutin (NAR) and didymin (DID) content in the juice samples before and after 24 h of fermentation at 37 °C. Graphs of means and LSD intervals for a confidence level of 95%.

3.2. Adhesion to Intestinal Epithelium after Fermentation as Affected by Trehalose Addition and/or Juice Homogenization

The adhesion to intestinal epithelium is one of the most important characteristics for a potentially successful probiotic. It is related to the microbial ability to colonize mucosal surfaces, to interfere with pathogen binding and to interact with the immune system cells [36], and so it is crucial for a probiotic strain to exert a beneficial effect on the host. To get adhered to the intestinal mucosa, probiotics have specific adhesion proteins on their surface that are recognized by the receptors of the epithelial cells in the small intestine. Aspecific interactions, such as hydrophobic interaction and aggregating interaction between surface macromolecules, are also involved in the colonization potential of probiotics and its contribution to the competitive exclusion of pathogens and to the improvement of the gut microbiome [37].

A critical first step in the development of most infections, bacterial adherence has become a target for the development of novel therapeutics and functional foods containing probiotics [38]. To assess the adhesive capacity of *Lactobacillus* to the intestinal mucosa, several in vitro model systems have been developed, including binding of bacteria to immobilized proteins and its quantitative measurement by fluorescent hybridization [39], as was used in this work. Figure 3 shows the capacity of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 strain to adhere to mucin (MUC), collagen (COL) and bovine serum albumin (BSA) as affected by the growing media (MRS broth or clementine juice formulated with and without 10% by weight of trehalose) and/or its homogenization at 100 MPa. Since they did not induce any stress in the microbial strain, HPH samples were not subjected to this analysis.

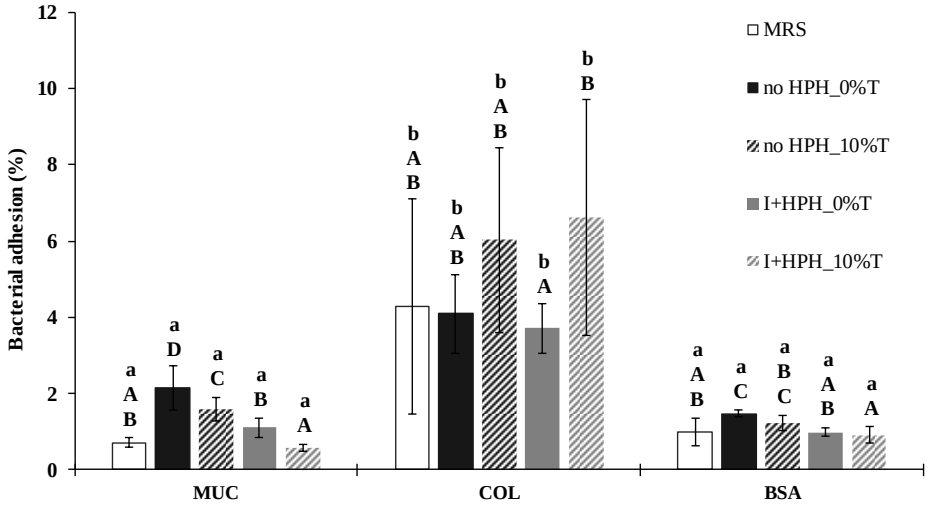

Figure 3. *L. salivarius* spp. *salivarius* CECT 4063 adhesion to mucin (MUC), collagen (COL) and bovine serum albumin (BSA) depending on the growth media composition and/or the homogenization at 100 MPa. Error bars represent the standard deviation of 6 replications. $^{a–b}$ For the same growing media and $^{A–D}$ for the same protein indicate statistically significant differences with a 95% confidence level ($p < 0.05$).

As can be seen, the percentage of cells that adhered to collagen was in all cases higher than that adhered to mucin or bovine serum albumin. This makes sense, since 70% of *Lactobacillus* isolates have been reported to express multiple adhesin types interacting with the collagen of the intestinal mucosa, which plays a decisive role in the competitive displacement of intestinal pathogens such as *Escherichia coli* 0157:H7 [40]. *Lactobacillus salivarius* spp. *salivarius* CECT 4063 attachment to the collagen surface was slightly lower than that reported by Rokana et al. [37] for *L. bulgaricus* NCDC26 (8.36 ± 1.47%) and *L. casei* ATCC393 (7.85 ± 1.72%), but considerably higher than that observed in *L. plantarum* NCDC20 (0.73 ± 0.52%) and *L. fermentum* NCDC214 (0.15 ± 0.03%). High deviations

obtained for adhesion to COL values suggest that non-specific interactions (such as hydrophobic interactions and aggregating interactions) might play a more important role in this case. Since such non-specific interactions are reported to be largely predisposed to the composition of bacterial surface covering components [40], slight modifications to the growing media composition or in the processing conditions might have a greater impact on them. Coupling between specific and non-specific interactions would also explain that the ability of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 to adhere to COL was in general terms higher than that to MUC.

Compared to MRS broth, the use of clementine juice (non-HPH_0%T) as growing medium increased the capacity of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 to adhere to any of the three proteins considered. This suggests that clementine juice is not only an excellent matrix for the growth of the microbial strain but also for the improvement of its probiotic properties. Neither the addition of trehalose to the juice before its inoculation (non-HPH_10%T), the homogenization at 100 MPa of the inoculated and 24 h-fermented juice (I+HPH_0%T) nor the combination of the two factors (I+HPH_10%T) significantly improved the ability of the microorganism to adhere to the proteins tested.

As regards the trehalose addition to the growing medium, it was previously reported to positively affect some properties of *Lactobacillus acidophilus* NCFM, such as bacteriocin production or growth rate, but not to significantly vary its adherence to mucin and HT-29 cells [41]. In the present study, trehalose significantly decreased ($p < 0.05$) the ability of *Lactobacillus salivarius* spp. *salivarius* CECT 4063 strain to adhere to both MUC and BSA. Only the microorganism adherence to COL was slightly increased by the addition of trehalose to the juice composition, and especially by further submitting the microbial strain to 100 MPa.

Regarding the application of high homogenization pressures, results obtained in this study also differ from those obtained by other authors [15,16] who confirmed that it can change the structure of microorganisms and increase their hydrophobicity, thus facilitating their adhesion to the cells of the digestive tract. Quite possibly, as documented by Patrignani et al. [42], response to HPH varies with the species and the characteristics of individual strains, as well as with the composition of the media containing the microorganism.

3.3. Storage of Fermented Clementine Juice

Changes in the microbial content of clementine juices fermented for 24 h with *Lactobacillus salivarius* spp. *salivarius* CECT4063 throughout 30 days of refrigerated storage at 4 °C were observed (Figure 4). HPH samples were not subjected to this study since the microorganism was not stressed by the application of a pressure gradient and so its tolerance to adverse conditions was expected to be similar to that of the microorganism that has grown in non-HPH samples. In agreement with previous findings [15], the number of living cells remained constant during the first 3 days of storage and began to decrease from the seventh day, probably due to nutrient and oxygen depletion and/or the excretion of organic acids and other biochemical contaminants into the medium. Zheng et al. [30] reported that *L. casei* was able to produce lactic acid even at refrigerated temperatures, thus reducing from 4.91 to 4.38 and from 5.31 to 4.48 the pH of fermented heat-treated lychee juice and fermented HHP-treated lychee juice, respectively, after storage for 28 days at 4 °C. In this study, no significant changes in the pH and Brix values of fermented clementine juice were observed after storage for 31 days at 4 °C ($p < 0.05$). It was not until day 21 when the counts reached values below the minimum concentration of 10^6 cfu/mL required to make an EU-based health claim [27].

Statistical analysis on the loss of viability that occurred between the beginning and the end of storage revealed, with a confidence level of 95%, that the non-HPH_0%T juice suffered the highest loss of viable cells (5.4 ± 0.5 $\log_{10}$ reduction on average). The simple addition of 10% (w/w) of trehalose to the juice formulation before its inoculation (non-HPH_10%T juice) slightly reduced the loss of viability to 4.92 ± 0.08 $\log_{10}$, regardless of the subsequent homogenization or not of the 24 h-fermented juice. On the contrary, the

homogenization at 100 MPa significantly decreased the loss of viability of *Lactobacillus salivarius* CECT 4063 in the juices that did not include trehalose in their formulation (I+HPH_0%T) in the order of 4.4 ± 0.5 $\log_{10}$. Similar results were obtained by Betoret et al. [15] after storage of clementine juice rich in *Lactobacillus salivarius* spp. *salivarius* for 10 days.

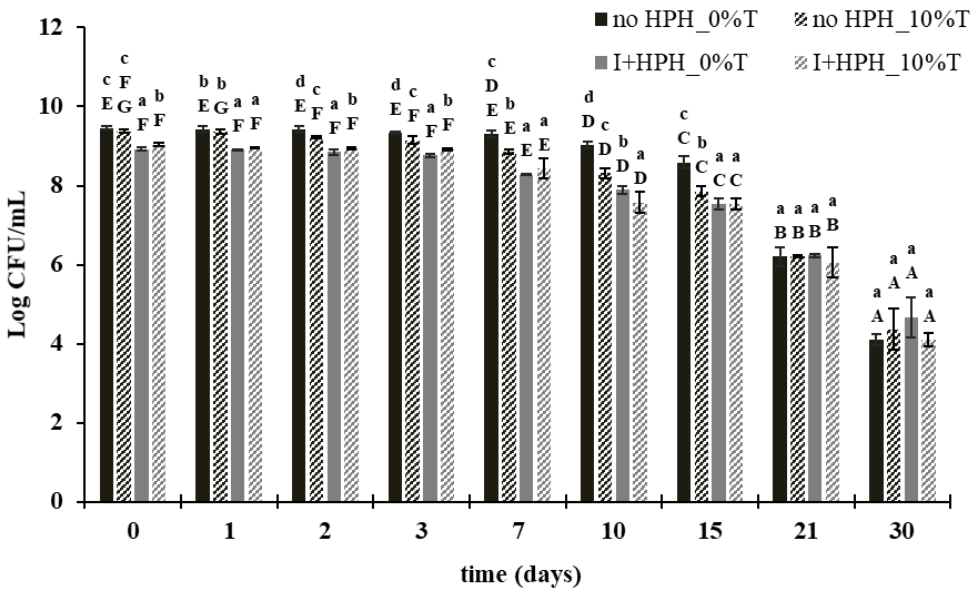

Figure 4. Microbial counts in clementine juice fermented with *L. salivarius* spp. *salivarius* CECT 4063 in cold storage for 30 days. Error bars represent the standard deviation of 4 replications. $^{a–d}$ In the same time and $^{A–G}$ in the same series indicate statistically significant difference with a 95% confidence level ($p < 0.05$).

Figure 5 shows the evolution in the antioxidant properties of 24 h-fermented juices during storage in refrigeration for 30 days. Total phenolics and total flavonoids ranged around a constant value, which turned out to be particular to each of the juices. A similar trend was observed for the ability to scavenge the radical $ABTS^{+}$, which, excluding the notable increase undergone within the first 24 h of storage and the drastic decline recorded on day 15, remained fairly stable. However, the antiradical activity measured by the DPPH method increased progressively throughout the storage period. This increase could be the result of the fermentative activity of the probiotic, which slowly continued under refrigeration conditions and allowed the formation of antioxidants compounds of a more hydrophobic nature and with a greater capacity to scavenge the radical $DPPH^{\bullet}$. In addition, the antioxidant capacity of phenolics could be enhanced by changes in their chemical structure taking place during the storage period [31].

Setting the end of the shelf life based on the microbial counts, 24 h-fermented juices should not be stored under refrigeration for more than 15 days in order to ensure the minimum concentration of 10^6 cfu/mL. Taking this into account, Table 3 shows the change in the antioxidant properties undergone by fermented clementine juices at the end of their shelf life. Results show that there was actually a significant decrease in all the antioxidant properties analyzed. Only the antioxidant activity measured by the DPPH method improved remarkably at the end of the shelf life. Decline in total phenolic content was also observed in fermented heat- and HHP-treated lychee juice [31] and in fermented and unfermented sweet lemon juice [43] during 4 weeks of storage at 4 °C and explained in terms of the oxidation degradation and the polymerization of phenolic compounds with proteins.

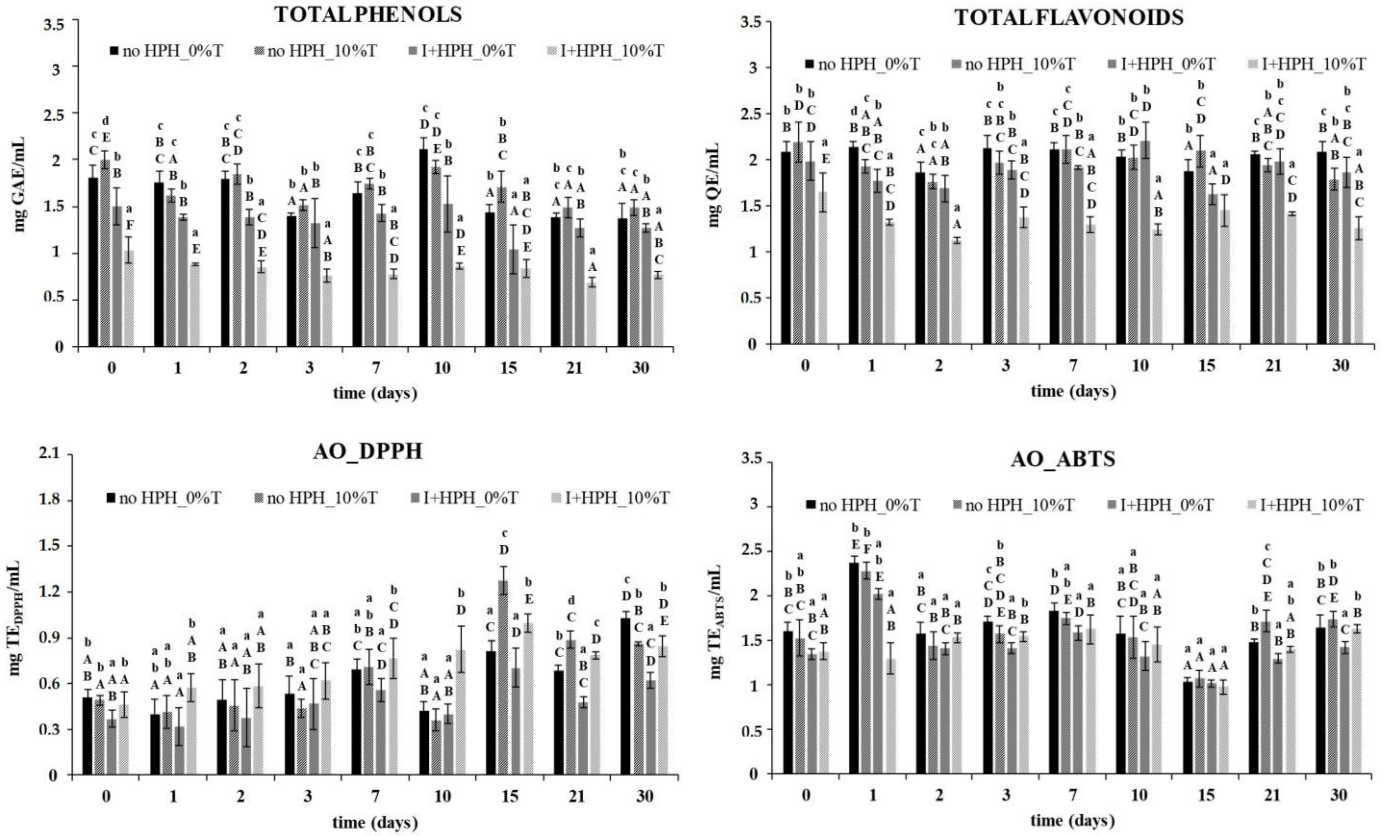

Figure 5. Antioxidant properties of clementine juice fermented with *L. salivarius* spp. *salivarius* CECT 4063 in cold storage for 30 days. Error bars represent the standard deviation of 4 replications. For each antioxidant property, [a–d] in the same time and [A–F] in the same series indicate statistically significant difference with a 95% confidence level ($p < 0.05$).

Table 3. Percentage of increase (values > 0) or decrease (values < 0) in the antioxidant properties under-gone by 24 h-fermented clementine juices stored for 15 days at 4 °C.

Treatment		Total Phenols	Total Flavonoids	AO_DPPH	AO_ABTS
non-HPH	0%T	-23.1 ± 0.8^{b}	-7.1 ± 0.6^{c}	55 ± 11^{a}	-36 ± 3^{a}
	10%T	-15 ± 8^{c}	-8 ± 2^{c}	155 ± 19^{c}	-27 ± 4^{b}
I+HPH	0%T	-39 ± 2^{a}	-21.0 ± 1.2^{a}	107 ± 15^{b}	-24 ± 3^{b}
	10%T	-14 ± 2^{c}	-14.3 ± 0.7^{b}	117 ± 13^{b}	-27 ± 4^{b}

[a–c] In the same column indicate statistically significant differences with a 95% confidence level ($p < 0.05$). Mean value of 4 replications ± standard deviation.

In general terms, degradation of both total phenols and total flavonoids was favored by the homogenization at 100 MPa of the juice after 24 h of fermentation. This can be due to the reduction in particle size that results from homogenization, so that phenolic compounds, including flavonoids, were more sensitive to the factors responsible for their deterioration. In addition, these compounds were probably more available to be used as a substrate by the lactobacillus strain in order to continue with the fermentation. Decline in both total phenols and flavonoids in I+HPH samples was significantly reduced by the addition of 10% (w/w) of trehalose to the juice formulation before its inoculation. Such a protective effect of trehalose was also observed in non-HPH samples for the total phenolic content, while the decrease in total flavonoid content was enhanced in the presence of that disaccharide.

Regarding the overall antioxidant capacity, the addition of trehalose, the homogenization and the combination of both factors resulted in a lower loss (as measured by the $ABTS^{+}$ radical method) or a greater increase (as measured by the $DPPH^{\bullet}$ radical method).

The improvement in the ability to scavenge the free radical DPPH$^{\bullet}$ observed in the juices after 15 days of cold storage was significantly more evident in non-HPH samples than those include trehalose in their composition. Homogenization of the 24 h-fermented juice reduced the protective effect attributed to trehalose on the total antioxidant capacity of the juices.

4. Conclusions

Fermentation of commercial clementine juice with *Lactobacillus salivarius* spp. *salivarius* CECT 4063 for 24 h has a positive impact on antioxidant properties, while conferring a potential probiotic effect. Extending fermentation reduces the microbial population and does not significantly improve the antioxidant content. Homogenizing the juice before inoculation is not recommended, since—despite it enhancing microbial growth—it favors antioxidant degradation. Adding 10% (*w*/*w*) of trehalose to juice formulation before inoculating and/or homogenizing the juice at 100 MPa after 24 h of fermentation has a negative impact on viable counts and does not significantly improve microbial strain ability to adhere to the proteins of the intestinal mucosa. However, both techniques have been proven to be effective in preventing antioxidant oxidation and cell decay during storage of fermented juice under refrigeration, which should not last more than 15 days. Overall, it would not be necessary to ad trehalose to the juice formulation or apply high homogenization pressures in order to get a 24 h-fermented liquid with maximum microbial and antioxidant contents throughout its shelf life.

Author Contributions: Conceptualization, N.B. and C.B.; methodology, N.B., L.S., C.B. and C.G.B.-B.; software, C.B.; validation, N.B., L.S. and C.B.; formal analysis, N.B., L.S. and C.B.; investigation, C.G.B.-B.; resources, C.B., N.B. and L.S.; data curation, C.G.B.-B.; writing—original draft preparation, C.B. and C.G.B.-B.; writing—review and editing, L.S., N.B. and C.B; visualization, N.B., L.S. and C.B.; supervision, C.B.; project administration, C.B.; funding acquisition, N.B., L.S. and C.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Generalitat Valenciana, project reference GV/2015/066 titled "Mejora de la calidad funcional de un snack con efecto probiótico y antioxidante mediante la incorporación de trehalosa y la aplicación de altas presiones de homogeneización."

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors would like to thank M. Carmen Collado, research leader at the Biotechnology Department of the Institute of Agrochemistry and Food Technology (IATA) of the Spanish National Research Council (CSIC) in Valencia, for providing us with the facilities to carry out the adhesion tests and her valuable contribution to the revision of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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