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Changes in antioxidant and probiotic properties of a freeze-dried apple snack during storage

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Abstract

This research developed an apple snack with potential probiotic effect (> 10^7 CFU/g) by combining vacuum impregnation with Lactobacillus salivarius spp. salivarius (CECT 4063) and freeze-drying. Throughout storage (30 days), both the lactobacillus viability and the total flavonoids content decreased. Trehalose adition (10% by weight) to the impregnation liquid and/or its homogenization at 100 MPa accelerated the loss of cell viability but delayed flavonoids degradation and promoted an increase in the amount of phenols and total antioxidants.

Keywords: *L. salivarius spp. salivarius; homogenization; trehalose; freeze-drying; antioxidants.*

1. Introduction

Helicobacter pylori is a pathogenic bacterium that causes severe gastric problems to a large part of the world's population, especially in less developed countries [1]. Traditional treatments based on antibiotics have side effects and are not 100% effective [2]. Recent studies show that some strains of the *Lactobacillus* genus are effective in the treatment against *Helicobacter pylori*, reducing the colonization of this pathogen, what has promoted the incorporation of *Lactobacillus* in the formulation of certain foods [3]. However, the survival of these microorganisms in food is rather limited, especially in not dairy products. Some food engineering techniques, such as the formulation with certain ingredients (e.g. probiotics) or the modification of the structures conferring protection to the microorganism (e.g. encapsulation) can be applied in order to alter probiotics functionality and/or increase their survival against adverse conditions. Specifically, in this study the effect of trehalose addition (10%, w/w) to the impregnation liquid and/or its homogenization at 100 MPa on *Lactobacillus salivarius* spp. *salivarius* (CECT 4063) survival during the manufacture and storage of a freeze-dried apple snack was evaluated. Given the high content in antioxidant compunds of the raw materials, also changes in this bioactive substances were analyzed.

2. Materials and Methods

2.1. Solid matrix

Apples (var. Granny Smith) cut into 5 mm thick rings (20 and 65 mm of internal and external diameter, respectively) were used as solid matrix for the snack preparation.

2.2. Impregnation liquids

The impregnation liquid was prepared from commercial clementine juice (Hacendado brand). Following the procedure described by Betoret et al. [4], yeast extract (5 g/L) and sodium bicarbonate (9,8 g/L) were added for the optimal microbial growth. When required, 100 g/kg of food grade trehalose (TREHA^{TM}, Cargill, Barcelona, Sapin) were also added to the juice formulation. Once all the ingredients were dissolved, the liquids were inoculated (10^9 CFU/L) with strain CECT 4063 of *Lactobacillus salivarius* spp. *salivarius* (Colección Española de Cultivos Tipo, Universitat de València, Burjassot, Spain) that had been previously grown on MRS Broth agar. After 24 h of incubation at 37 ºC, part of the liquids were homogenized at 100 MPa on a laboratory scale high pressure homogenizer (Panda Plus 2000, GEA-Niro Soavi, Parma, Italy) before their use as impregnation liquids. Four different impregnation liquids were prepared in total (Table 1).

Table 1. Different impregnation liquids employed in the present study.

Impregation liquid	Trehalose (g/kg)	Pressure (MPa)
0%_0MPa	0	0
0%_100MPa	0	100
10%_0MPa	100	0
10%_100MPa	100	100

2.3. Experimental procedure

This section describes the unit operations involved in the manufacture of apple snacks enriched with *Lactobacillus salivarius* spp. *salivarius* (CECT 4063).

First, vacuum impregnation was performed in a vacuum chamber (HERAEUS Vacuun Oven, THERMO SCIENTIFIC) connected to a vacuum pump (ILMVAC, Germany). A vacuum pressure of 50 mbar was applied for 10 min to the apple rings immersed in the corresponding liquid. Then, the atmospheric pressure was restored and maintained for another 10 min.

Vacuum impregnated apple rings were deep-frozen and kept at -40 °C for 24 h (Matek model CVN-40/105). Then, they were placed in a pilot scale freeze-drier (TELSTAR LIOALFA 6-80) at -45 °C and a vacuum pressure of 0.1 mbar for 24 h more.

In the end, freeze-dried apple slices were stored in hermetic and opaque bags and kept under controlled conditions of humidity and temperature for 30 days.

2.4. Analytical determinations

All the analytical determinations were carried out at least in triplicate on liquid and/or solid samples at different stages throughout the snack manufacture process.

2.4.1. Water content and water activity

The apple samples moisture content was determined by drying a known amount of sample in a vacumm oven at 60 ºC and 200 mbar until it reached a constant weight.

The water activity of apple samples was measured at 25 °C in a properly calibrated dew poin hygrometer (Decagon Aqualab model CX-2, with an accuracy of ± 0.003).

2.4.2. Antioxidant properties

Extracts from solid samples were obtained by mixing 2 g of fresh and impregnated apple or 0.35 g of freeze-dried apple with 10 mL of a 80:20 (v/v) methanol-water solution.

Total phenols content was measured at 760 nm in a Helios Zeta UV/Vis Thermo Scientific spectrophotometer by the Folin-Ciocalteu reagent method [5]. Results were expressed in milligrams of gallic acid equivalents per gram of sample (mg GAE/g).

Total flavonoids content was measured at 368 nm in a Helios Zeta UV/Vis Thermo Scientific spectrophotometer by the colorimetric method of aluminum chloride [6]. Results were expressed in milligrams of quercetin equivalents per gram of sample (mg QE/g).

Antioxidant activity was determined at 515 nm in a Helios Zeta UV/Vis Thermo Scientific spectrophotometer by the DPPH method [7]. Results were expressed in milligrams of trolox equivalents per gram of sample (mg TE/g).

2.4.3. Viable counts

The *Lactobacillus salivarius* spp. *salivarius* (CECT 4063) concentration was analyzed both in the growing medium, the impregnation liquids and the apple samples by the serial dilution and plating method. MRS seeded plates were incubated in anaerobiosis at 37 °C for 3 days. In the case of solid apple samples, the first dilution was carried out in a stomacher bag in which 5 g of sample were crushed with 45 mL of sterile peptone water.

2.5. Statistical analysis

The effect that the different variables considered exert on the obtained results was evaluated with the Statgraphics Centurion XVI program by simple analysis (simple ANOVA) with a 95% confidence level.

3. Results and discussion

3.1. Changes in *Lactobacillus salivarius* spp. *salivarius* (CECT 4063) content

As it is shown in Fig. 1, *Lactobacillus salivarius* spp. *salivarius* (CECT 4063) content in the impregnation liquid (VI liquid) increased significantly by adding 10% of trehalose by weight to its formulation or by its homogenization at 100 MPa. However, the combination of both factors did not notably improve the viable counts.

Similar trends were observed in vacuum impregnated apples (VI apple), whose microbial content was significantly lower than that of any of the impregnation liquids. This is logical considering that only 20% of fresh apple volume is filled with the impregnation liquid during the vacuum impregnation step [8].

Finally, freeze-dried apple samples (FD apple) potential probiotic effect was higher than that of vacuum impregnated ones, but not as high as expected from the decrease in their water content (from 85.3 ± 1.2% in VI apple to 4.97 ± 1.02% in FD apple). Regarding the composition of the impregnation liquid, the addition of trehalose to its composition slightly reduced the adverse effect of freeze-drying on the microbial population. On the contrary, the subsequent homogenization of trehalose enriched juice increased *L. salivarius* spp. *salivarius* vulnerability to the freeze-drying step.

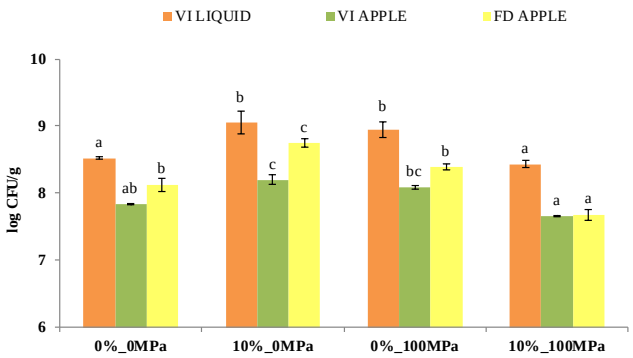

Fig. 1. Microbial counts in the impregnation liquids and both in vacuum impregnated (VI) and freeze-dried (FD) apple samples. Different letters within a single series indicate statistical significant differences at 95% confidence level.

Throughout storage (Fig. 2), *L. salivarius* spp. *salivarius* (CECT 4063) content in freeze-dried apple samples suffered a notable decline, in spite of the low water activity reached by the snack (0.27 ± 0.02). This fact was particularly evident when trehalose and/or pressure were applied to the impregnating liquid, thus suggesting that the stress caused by the osmotic and/or the pressure gradient favored the loss of vialbility and the shortening of the snack self life. Just to mention that for a food to be considered probiotic it must contain at least 10^7 CFU/g when consumed [9]. Only snacks impregnated with liquids 0%_0MPa and 10%_0MPa met this condition at the end of the storage.

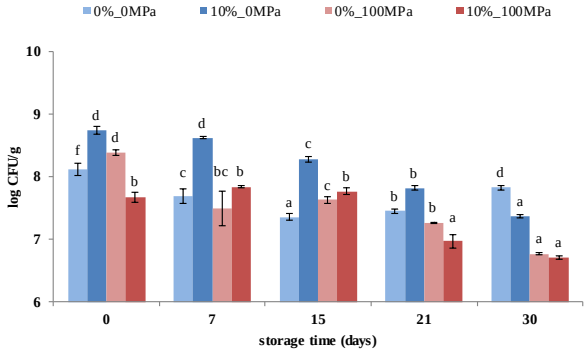

Fig. 2. Changes in microbial counts troughout storage of freeze-dried apple snacks. Different letters within a single series indicate statistical significant differences at 95% confidence level.

3.2. Changes in antioxidant properties

Despite the notable differences observed in their total phenols and flavonoids content, antioxidant capacity assessed by DPPH method both in the impregnation liquids and the vacuum impregnated apple samples were of the same order (Table 2). As expected, all the antioxidants increased their concentration significantly after the freeze-drying step. As for the composition of the vaccum impregnation liquid, neither the addition of trehalose nor the homogenization resulted in a final snack with improved antioxidant properties.

Table 2. Antioxidant properties of the impregnation liquids and both the VI and FD apple samples.

Food matrix	Impregation liquid	total phenols (mg GAE/g)	total flavonoids (mg QE/g)	DPPH (mg TE/g)
VI liquid	**0%_0MPa**	0.78(0.07)^{a}	1.01(0.07)^{c}	0.70(0.04)^{a}
	10%_0MPa	0.79(0.08)^{a}	0.904(0.004)^{c}	0.78(0.06)^{a}
	0%_100 MPa	0.7235(0.0007)^{a}	0.954(0.014)^{c}	0.7(0.2)^{a}
	10%_100 MPa	0.82(0.03)^{a}	0.93(0.03)^{c}	0.73(0.05)^{a}
VI apple	**0%_0MPa**	0.441(0.015)^{a}	0.291(0.002)^{b}	0.89(0.12)^{a}
	10%_0MPa	0.34(0.06)^{a}	0.175(0.003)^{a}	0.61(0.03)^{a}
	0%_100 MPa	0.82(0.11)^{a}	0.217(0.002)^{ab}	0.92(0.07)^{a}
	10%_100 MPa	0.64(0.03)^{a}	0.132(0.002)^{a}	0.77(0.04)^{a}
FD apple	**0%_0MPa**	11.4(0.6)^{e}	8.70(0.07)^{f}	7.84(0.07)^{d}
	10%_0MPa	3.025(0.012)^{b}	1.186(0.012)^{d}	5.5(0.2)^{c}
	0%_100 MPa	6.0(0.3)^{d}	2.01(0.14)^{e}	5.0(0.3)^{c}
	10%_100 MPa	4.8(0.4)^{c}	1.19(0.02)^{d}	2.3(0.8)^{b}

Mean values and standard deviation in brackets. Different superscripts in the same column indicate statistical significant differences at 95% confidence level.

Regarding the stability of the antioxidant compounds throughout the snack storage, it is shown in Fig. 3 the change in each component concentration from the beginning to the end of the storage referred to the initial concentration (Δx^i):

$$\Delta x^i(\%) = \frac{x^i_{t=0} - x^i_{t=30}}{x^i_{t=0}} \cdot 100 \quad (1)$$

where $x^i_{t=0}$ and $x^i_{t=30}$ indicate the component i concentration in freshly made and 30 days stored FD apples, respectively (g of component i/total g).

Generally speaking, the total phenols gain was significantly higher and the total flavonoids loss was significantly lower (p-value < 0.05) in apple snacks that were impregnated with any of the liquids that included trehalose in its composition, but especially in those impregnated with the liquid that was additionally homogenized (10% _100MPa). As a result, these samples also showed a significantly higher increase in the total antioxidant content measured by the DPPH method.

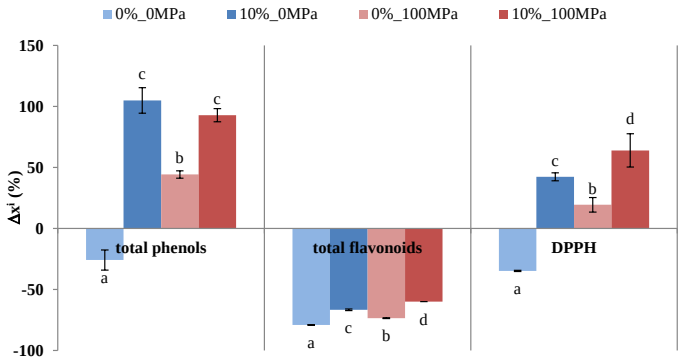

Fig. 3. Changes in antioxidant properties after 30 days of storage of freeze-dried apple snacks. Different letters within a single compound indicate statistical significant differences at 95% confidence level.

Given the ability of trehalose to protect biological structures [10], apple tissue would have been less damaged during the freeze-drying step. As a result, antioxidant compounds would have been less accessible to adverse conditions that promote their degradation. Trehalose protective effect could be promoted by the reduction in the particle size that implies the application of a homogenization step [11], thus favouring the inflow of a greater amount of liquid (and trehalose) into the apple porous structure during the vacuum impregnation.

4. Conclussions

Vacuum impregnation allows to incorporate lactobacillus into the apple porous structure to a greater or lesser extent, depending on the viable counts in the impregnation liquid. The subsequent freeze-drying increases apples stability without negatively affecting the microbial content or the antioxidants content. *Lactobacillus salivarius* spp. *salivarius* survival during the further storage was negatively affected by the addition of trehalose to the impregnation liquid and/or its homogenization. On the contrary, the addition of trehalose to the impregnation liquid and/or its homogenization delayed flavonoids degradation and promoted an increase in the amount of both phenols and total antioxidants.

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