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Vacuum Impregnation of Probiotics in Fruit Pieces and Their Survival During Refrigerated Storage

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ABSTRACT

Even though probiotics are currently available mainly in dairy products, interest in the incorporation of probiotics in other foods has been increasing. This research aimed to develop probiotic foods by fortifying the probiotics in partially-dried fruits using a vacuum impregnation technique. Fruit (guava and papaya) pieces were impregnated under a vacuum pressure of 50 mBar with three types of extracted fruit-juice solutions: 15°Bx extracted and 30°Bx extracted fruit juices containing 10^{10} cfu/mL of *Lactobacillus casei* 01 for 5, 10 and 15 min, respectively. After impregnation, the fruit samples contained amounts of the probiotics ranging from 10^8 to 10^9 log cfu/g. The impregnation time and the soluble-solid contents of the impregnated solution affected vacuum impregnation parameters such as the impregnated sample volume fraction (X) and the effective porosity (ε_e). No change or only a slight change in the volumetric deformation (γ) of the fruit pieces occurred after impregnation. The soluble-solid contents also influenced the level of probiotics in the products. If the amount of soluble solids was either too low or too high, then the viable count of the probiotics was reduced. In order to increase the storage stability of the products, the impregnated guavas and papayas, which had been impregnated with fruit juice containing 15°Bx for 10 and 5 min, respectively, were dried at 40°C for 36 h and kept at 4°C for four weeks. The viable cell counts of *L. casei* 01 in both impregnated guavas and papayas were approximately 10^7 log cfu/g, which reached the therapeutic minimum level of dairy products.

Key words: probiotics, vacuum impregnation, fruit pieces, papaya, guava

INTRODUCTION

Probiotics are defined as live microorganisms that beneficially affect the host by improving its intestinal microbial balance. Probiotic bacteria in foods have been used to promote health benefits for 20 years. Their benefits include: controlling intestinal infection, controlling serum cholesterol levels, beneficially influencing the immune system, improving lactose utilization in lactose maldigestors and exhibitting anticarcinogenic activity (Noh and Gillilnad, 1993; Kailasapathy and Rybka, 1997; Berner and O Donnell, 1998; Guandalini *et al*., 2000; McNaught and MacFie, 2001; Saarela *et al*., 2002; Ouwehand *et al*., 2003; Rafter, 2003). To achieve optimal beneficial effects, the amount of probiotic bacteria in the product should be at least 10^7 cfu/mL (Robinson, 1991; Ouwehand and Salminen, 1998). Currently, industrial probiotic food products have been mainly added to dairy products such as yogurt and fermented milk. However, lactose intolerance

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and the cholesterol content in milk are two consumer-identified drawbacks related to the consumption of probiotics in dairy products. Hence, partially-dried fruit containing probiotics would provide a new option for those who cannot consume dairy products. In addition, the partially-dried fruits would be rich in nutrients such as vitamins and minerals. The product would also contain a high level of natural antioxidants.

Vacuum impregnation (VI) is one method used for the fortification of probiotics into fruit matrices and involves the application of a partial vacuum as the driving force to diffuse water from the tissue under the higher osmotic pressure of the hypertonic solution (Fito, 1994; Fito *et al*., 1996; Guerrero, 1996; Rastogi and Raghavarao, 1996; Salvatori, 1997; Martflnez-Monz¸ *et al*., 1998; Salvatori *et al*., 1998). It was considered to be a technique that could quickly introduce external liquids into the porous structure of animal and plant tissues in a controlled way. As a consequence, some mass transfer processes have been improved and some changes in food composition produced (Zhao and Xie, 2004).

The vacuum impregnation process consisted of the application of reduced pressure to a solid-liquid system, followed by the restoration of atmospheric pressure (Fito, 1994). The decline in pressure caused an increase in the escape of gas in the pores. When the pressure was restored, the pores were occupied by the osmotic solution and the available mass transfer surface area was increased (Li and Ramaswamy, 2005). The quantity of liquid impregnated into the food structure during the vacuum impregnation depended on the vacuum pressure (VP) and the application time. Manipulation of the vacuum pressure allowed for a more rapid and controlled impregnation of the desired solute into foods. It has been applied to minimally processed fruit (Tapia de Daza *et al*., 1996).

Rodriguez (1998) reported that the lower the absolute pressure of the vacuum pulse applied, the higher the incorporation of *Lactobacillus acidophilus* in Granny Smith apples using isotonic sucrose as the impregnation solution. Moreover, apple cylinders fortified with *Bifidobacterium* spp. "Bb12" (Christian Hansen Corp.) contained the bifidobacteria at a level of approximately 10^8 cfu/g at an absolute pressure of 101 to 125 mmHg (Maguina *et al*., 2002). During the anaerobic storage of apple pieces at 4°C for 12 d, there was a log-decline in the number of the probiotics after the sixth day and the number of probiotics remained at this level until the end of storage period.

Ortiz *et al*. (2002) studied the supplement of *Bifidobacterium* sp. in guava using VI at a high vacuum pressure of 400 mmHg for 5 min. The impregnated guava pieces contained about 10^7 cfu/g. The viable count of the products reduced three logs after 12 d of storage at 5°C due to aerobic packing. Betoret *et al*. (2003) used a low vacuum pressure of 38 mmHg to impregnate *Lactobacillus casei* ssp. *rhamnosus* CELT 245 into apple pieces using commercial apple juice or whole milk as the impregnated solution and successfully replaced gas with the impregnated liquid. Lapsley *et al*. (1992) found that when the size of the intracellular space was between 210-350 åm, the microbial cells present in the impregnation liquid were able to enter the intracellular spaces. After drying for 48 h at 40°C, the level of *L. casei* in the finished product was 10^8 cfu/g and the probiotic content decreased by less than one log after storage at 20°C for 15 days. The product also contained a moisture content of 3.7-4.4%, which may have had an effect on its texture.

Therefore, this research aimed to study the feasibility of vacuum impregnation to incorporate probiotic bacteria into fruit pieces and also to investigate the survival of probiotics in partially-dried fruit products.

MATERIALS AND METHODS

Structural and physico-chemical analysis of raw materials

The physico-chemical properties and the porosity of the raw materials were characterized. A refractometer (Tamco, Japan) was used to determine the content of soluble solids. A pH meter (Hunna instrument pH211 microprocessor pH meter) was used to measure the pH. Fruit acidity was titrated with NaOH 0.1 N, using phenolphthalein as a color indicator and was expressed as a weight percentage of citric acid. The ripeness index for each fruit was calculated as the ratio of the soluble-solids content to acidity. The moisture content was determined using the AOAC standard method, 20.013 (AOAC, 1984). The pycnometer method, using an isotonic solution as a reference liquid, was used to determine the apparent density (ρ_a) and the solid/liquid density or real density (ρ_r). Fruit porosity or real porosity (ε_r) was calculated from these values by means of Equation (1) (Salvatori *et al*., 1998).

$$\varepsilon_r = \frac{(\rho_r - \rho_a)}{\rho_r} \quad (1)$$

Preparation of impregnation liquid

A slant of *L. casei* 01 (LC01) (Chr. Hansen Pty Ltd.) was inoculated into 10 mL of three types of impregnation liquid. The first type consisted of natural fruit juices (papaya and guava) that were extracted by blending with water using a ratio of fruit to water of 1:1 by weight. The second and the third types were extracted fruit juices containing 15 and 30°Bx respectively. The pH of the juices was maintained within the range of 5.8-6.0 by adding 5 g/L of sodium bicarbonate. The juices were then incubated at 37°C for 48 h under aerobic conditions. The cultures were then transferred into 95 mL of the fruit juice and then incubated under the same conditions. These juices were used as impregnation liquids.

Fruit impregnation and air drying

The method of vacuum impregnation was derived from Betoret *et al*. (2003). Papaya and guava were peeled and cut into cylindrically-shaped samples (40 mm length and 18 mm diameter) with reference to their vertical axis. The fruit pieces were immersed in the three types of impregnation liquid as described above. A vacuum pressure of 50 mbar was applied for 5, 10 and 15 min and then atmospheric pressure was restored leaving the samples under the liquid for an additional 10 min. The samples were weighed at the beginning and the end of impregnation process to determine the amount of liquid incorporated into the fruit slices (the impregnated sample volume fraction, *X*). The volume of the samples was measured at the end of vacuum impregnation to determine the volumetric deformation of the sample (γ) using Equation (2), where ν_0 was the initial volume of samples and ν_t was the final volume of samples (Salvatori *et al*., 1998).

$$\gamma = \frac{\nu_t - \nu_0}{\nu_0} \quad (2)$$

The effective porosity (ε_e) of the fruits was calculated using Equation (3). The compression ratio (r) was calculated using Equation 4 as a function of the pressure in the chamber in the periods t_1 and t_2. The capillary term (p_c/p_1) can be neglected for values of vacuum pressure lower than 400-600 mbar (Salvatori *et al*., 1997).

$$X - \gamma = \varepsilon_e\left(1 - \frac{1}{r}\right) - \frac{\gamma}{r} \quad (3)$$

$$r = \frac{p_2 + p_c}{p_1} \quad (4)$$

The fruit samples with the highest number of probiotics after vacuum impregnation were then dried for 36 h in a cabinet dryer at 40°C. After drying, the samples were kept in plastic bags at 4°C for four weeks.

Microscopic examination

Scanning Electron Microscopy (SEM) was used to investigate the location of the probiotic cells in the fruit tissue after impregnation. A transverse section from a slice, which was taken from the middle section of a cylinder, was excised, mounted in stainless steel stubs, gold coated and observed by SEM (JEOL JSM-6310F).

Chemical and microbiological analysis

Enumeration of the probiotic bacteria was determined after vacuum impregnation and during storage using MRS (de Man, Rogosa, Sharpe) agar (Oxoid). The moisture content of the products was also evaluated by the AOAC 20.013 method (AOAC, 1984) during storage**.**

Statistical analysis

The experiment was set up using a 3 × 3 symmetrical, factorial, randomized block design (RBD), using fruit as the block, with three replications. Mean differences and Duncan's New Multiple Range Test were analyzed using SPSS for Windows 14.0.

RESULTS AND DISCUSSION

Physico-chemical characteristics of guava and papaya

The physico-chemical characteristics of guava and papaya used in this experiment are presented as mean values with a standard deviation (SD) in Table 1. The low SD values in most cases reflected the homogeneity of the selected batches of fruits. The ripeness index corresponded to fruits with a firm texture and good organoleptic characteristics (Primo-Yufera, 1982). The ripeness index of guava and papaya was 35.96 and 79.92 respectively. Fruit porosity or real porosity constituted a measure of the empty space in the fruit tissue and represented the maximum space that could be impregnated with the solution. Guava tissue contained less empty space than papaya tissue, as the real porosity or fruit porosity of guava (7.597%) was less than for papaya (13.848%).

Microscopic observation of microorganism location in the impregnated samples

The results of the microscopic observation using SEM of the guava and papaya pieces following the impregnation treatment are shown in Figure 1. The electron micrographs indicated that *L. casei* 01 cells were immobilized on the guava and papaya pieces. This implied that the size of the intercellular spaces of guava and papaya tissues were large enough to allow the cells of *L. casei* 01 to pass through. Cell sizes in the range 0.7-1.1 × 2.0-4.0 μm were able to pass through these spaces (Axelsson, 1998).

Vacuum impregnation parameters and the number of probiotics after vacuum impregnation of guava and papaya

Table 1 Physico-chemical characteristics of guava and papaya.

Characteristics	Guava	Papaya
pH	3.55 ± 0.09*	5.32 ± 0.01
Soluble solids (°Bx)	9.9 ± 0.1	9.3 ± 0.5
Fruit acidity (%)	0.28 ± 0.01	0.12 ± 0.01
Ripeness index	35.96 ± 1.87	79.92 ± 1.39
Moisture content (%)	83.6 ± 0.2	92.8 ± 0.5
Apparent density (kg/m^3)	990 ± 30	1010 ± 70
Real density (kg/m^3)	1080 ± 60	1180 ± 80
Fruit porosity (ε_r) (%)	7.597 ± 3.222	13.848 ± 2.119

* Mean ± standard deviation

Guava and papaya pieces were impregnated using three types of liquid that included natural fruit extract or fruit juices containing 15 and 30°Bx, at a vacuum pressure of 50 mbars for 5, 10 and 15 min. Vacuum impregnation parameters, such as the impregnated sample volume fraction (X), effective porosity (ε_e) and the number of probiotics, were determined after impregnation (Table 2). The results showed that the impregnation time and the soluble-solid contents of the impregnated solution had significantly affected ($p \leq 0.05$) the impregnated sample volume fraction (X) and the effective porosity (ε_e). On the other hand, no change or only a slight change occurred in the volumetric deformation (γ) of the fruit pieces after impregnation. This might have been caused by their rigid cellular structure and their wide intercellular pores, which offered little resistance to the fluid flow. Therefore, the volume of the fruit pieces remained the same or almost the same as before vacuum impregnation.

The X value refers to the volumetric fraction of the sample occupied by the external liquid. In the case of guava, the X value noticeably increased when the vacuum time increased from 5 to 10 min (from 0.694 to 1.019 at 4°Bx, for example) and then slightly decreased when the fruits were impregnated for 15 min (0.907). This result implied that the longer the time under vacuum, the higher the volume of impregnated solution until the structure of guava was partially deformed at 15 min, resulting in a reduction of X. Conversely, there was a reduction of X in the case of papaya with an increase in the vacuum time. This was probably caused by an irreversible partial-deformation of the porous structure, which could then not hold the impregnated solution and the texture of the papaya was also softer than that of the guava (lower ripeness index). The X value was also affected by the concentration of the impregnated solution. As the soluble-solid contents increased, the X value tended to decrease in all treatments. This might have been caused by the higher viscosity of the solution due to the increase in the soluble-solid contents, resulting in a slower flow of the solution into the fruit tissue.

The effective porosity (ε_e) was considered an important parameter that could be used to describe the sample behavior during vacuum impregnation, because it determined the volume in the product tissue that could be occupied by the external liquid (Fito and Pastor, 1994). The ε_e obtained from this experiment paralleled the results for the X value, which was converse to the results obtained by Fito *et al*. (1996), who reported

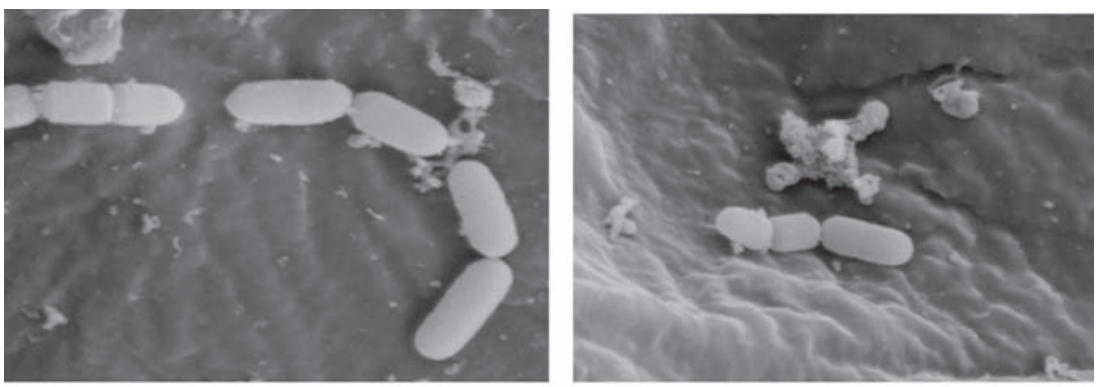

Figure 1 Scanning electron micrographs of *Lactobacillus casei* 01 on (a) guava and (b) papaya at 15,000x magnification.

Table 2 Effective porosity, volumetric impregnation parameters and the number of probiotics for guava and papaya pieces after vacuum impregnation in guava and papaya juice containing various total solid contents.

Fruit	Time (min)	ε_e*			*X*			No. of probiotics (Log cfu/g)		
		4°Bx	15°Bx	30°Bx	4°Bx	15°Bx	30°Bx	4°Bx	15°Bx	30°Bx
Guava	5	0.730±0.089^{c**}	0.465±0.005^{ab}	0.297±0.012^{a}	0.694±0.085^{c}	0.442±0.004^{ab}	0.283±0.012^{a}	8.94±0.57^{ab}	9.59±0.07^{c}	8.58±0.14^{ab}
	10	1.071±0.154^{d}	0.592±0.058^{bc}	0.424±0.075^{ab}	1.019±0.147^{d}	0.564±0.056^{bc}	0.404±0.071^{ab}	8.90±0.12^{b}	9.71±0.03^{c}	8.65±0.75^{ab}
	15	0.954±0.191^{d}	0.555±0.023^{ab}	0.349±0.035^{a}	0.907±0.181^{d}	0.527±0.022^{b}	0.332±0.033^{a}	9.62±0.09^{c}	9.62±0.01^{c}	8.49±0.02^{a}
Papaya	5	0.498±0.191^{c}	0.519±0.049^{c}	0.249±0.037^{a}	0.473±0.182^{c}	0.494±0.047^{c}	0.237±0.036^{a}	9.33±0.44^{bc}	9.56±0.12^{bc}	8.60±0.15^{a}
	10	0.479±0.016^{c}	0.403±0.017^{bc}	0.238±0.026^{a}	0.455±0.015^{c}	0.383±0.016^{bc}	0.226±0.025^{a}	9.70±0.19^{c}	9.63±0.01^{bc}	8.57±0.06^{a}
	15	0.467±0.053^{c}	0.398±0.021^{bc}	0.289±0.049^{ab}	0.444±0.050^{c}	0.378±0.020^{bc}	0.275±0.047^{ab}	9.66±0.12^{bc}	9.56±0.09^{bc}	9.57±0.09^{bc}

* ε_e = effective porosity, *X* = volumetric impregnation parameter

** The same letter for both a row and column means no significant difference at the 95% confidence level. The statistical analysis of each fruit was done separately.

that the experimental ε_e value was practically constant for most of the fruits and vegetables sampled when the pressure was below 600 mbar. A comparison of the fruit porosity (ε_r) and the effective porosity (ε_e) of the studied fruits (Tables 1 and 2) showed that ε_r was greater than ε_e in both the guava and papaya samples. This indicated that there was still free volume available for impregnation. Nevertheless, due to the capillary effects or structural modifications, the free volume was not completely filled (Andrés, 1995). The $\varepsilon_e/\varepsilon_r$ ratio can be defined as the total fraction of the pores available for hydrodynamic mechanisms (HDM). The $\varepsilon_e/\varepsilon_r$ ratio was within the range of 0.039-0.141 and 0.015-0.037 for guava and papaya respectively. This ratio showed that the guava had more pores available for HDM than the papaya, although papaya had higher fruit porosity than guava.

The probiotic contents of all treatments are also shown in Table 2. The number of probiotics in all treatments was as high (8.0 log cfu/g) as the results obtained from the study of Betoret *et al.* (2003) where *L. casei* ssp. *rhamnosus* was impregnated into apples using apple juice as the impregnated solution. For guava at 4°Bx, the number of probiotics increased when the vacuum time increased (from 8.94 to 9.62 log cfu/g), while there were significant differences ($p \leq 0.05$) between 15 and 30°Bx. Moreover, there was a reduction of the probiotic content at 30°Bx in all experiments. This was probably due to a bacterial inhibitory effect caused by the high concentration of sugar. For papaya, the number of probiotics in all treatments (9.60-9.70 log cfu/g) was not significantly different ($p>0.05$), except for the papaya impregnated at 30°Bx for 5 and 10 min, which had a lower number than the others (8.57-8.60 log cfu/g). In conclusion, suitable conditions for the impregnation of guava and papaya were impregnation in fruit juice containing 15°Bx for 10 and 5 min, respectively.

Table 3 Number of probiotics (log cfu/g) in partially-dried fruits stored at 4°C for four weeks.

Type of fruit	Storage time (week)				
	0	1	2	3	4
Guava	8.52±0.38*	9.09±0.26	8.41±0.46	7.50±0.36	7.17±0.49
Papaya	8.85±0.52	9.18±0.09	8.49±0.26	8.30±0.2	7.52±0.36

* Mean ± standard deviation

Storage of vacuum impregnated and air dried fruits

After vacuum impregnation, the samples were air dried at 40°C for 36 h and then kept in a refrigerator (4°C) for four weeks. The results are shown in Table 3. The number of probiotics increased after drying from 8.09 to 8.52 log cfu/g and 8.56 to 8.85 log cfu/g for guava and papaya respectively. After one week of storage, the number of probiotics significantly increased ($p \leq 0.05$) from 8.52 to 9.09 log cfu/g for guava and from 8.85 to 9.18 log cfu/g for papaya. This result implied that the environment inside the fruits was favorable for the growth of *L. casei* 01. After more than one week of storage, the number of probiotics dropped slightly to 8.41 and 8.49 log cfu/g and continued to decline to 7.17 and 7.52 log cfu/g at the end of storage for guava and papaya, respectively. However, these levels were higher than the therapeutic level (7.0 log cfu/g) for probiotics in dairy products. The probiotic level in partially-dried fruits was also similar to the results obtained from the study by Maguina *et al*. (2002). They reported that the product with *Bifidobacterium* sp. stored at 4°C for 12 d, contained a high number of probiotics (8.0 log cfu/g). Betoret *et al*. (2003) also demonstrated that impregnated dried apple stored at 20°C for 15 d contained approximately 7.0 log cfu/g *of L. casei* ssp. *rhamnosus*.

CONCLUSION

The impregnation time and the soluble-solid contents of the impregnated solution affected vacuum impregnation parameters such as the impregnated sample volume fraction (χ) and the effective porosity (ε_e). No change, or only a slight change, in volumetric deformation (γ) of the fruit pieces occurred after impregnation. The soluble-solid contents also influenced the level of probiotics in the products. If the amount of solid contents was either too low or too high, then the viable count of the probiotics was reduced. Suitable conditions for the impregnation of guava and papaya were vacuuming fresh guava and papaya with 15°Brix fruit juice for 10 and 5 min respectively. In order to increase the storage stability of the products, the impregnated guava and papaya were dried at 40°C for 36 hrs and kept at a refrigerated temperature of 4°C for four weeks. The viable cell count of *L. casei* 01 in both guava and papaya was approximately 10^7 cfu/g, which reached the therapeutic minimum level in dairy products.

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