No invasive methodology to produce a probiotic low humid apple snack with potential effect against *Helicobacter pylori*.

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Abstract

A probiotic low humid apple snack with potential effect against the infection caused by *Helicobacter pylori* has been developed from apple (cv. Granny Smith) and mandarin juice with a high microbial content of *Lactobacillus salivarius* spp. *salivarius*, by vacuum impregnation and hot air drying techniques. The moisture content reached in the final product (0.144 ± 0.012 g\textsubscript{water}/g\textsuperscript{1} sample) ensured stability, and although the drying process affected the microbial content, the concentration in the final product (9.486 ± 0.013)\cdot10\textsuperscript{7} CFU/g\textsuperscript{1} dry sample) was sufficient to confirm that with this procedure it is possible to obtain a stable probiotic fruit with a low moisture content. Additionally, a preliminary in vivo test with five dyspeptic children was undertaken that suggested the possible effect of this new product on *Helicobacter pylori* as measured by a standard infection indicator.

Key words: probiotic, *Lactobacillus salivarius* spp. *salivarius*, mandarin juice, pineapple/grape juice, vacuum impregnation, *Helicobacter pylori*.

1. INTRODUCTION

Probiotics are defined as live microbial food ingredients that have a beneficial effect on human health (Salminen et al., 1999) or as live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance (Fuller, 1989). Scientific evidence suggests that probiotic bacteria, consumed at high levels (10\textsuperscript{9}–10\textsuperscript{11} CFU per day) can decrease the incidence, duration, and severity of some intestinal illnesses (Sanders, 1999). For example, it has been shown that *Lactobacillus salivarius* suppress *Helicobacter pylori* and reduces the inflammatory responses in gnobiotic mice better than *L. casei* (Aiba et al., 1998) and *L. acidophilus* has been demonstrated to inhibit *H. pylori* better than other strains (Mrda et al., 1998; Canducci et al., 2000; Sheu et al., 2002). *H. pylori* is an etiologic agent of chronic gastritis and gastroduodenal ulcers and its inclusion by the International Agency for Research on Cancer in 1994 as a carcinogenic agent type 1, has turned it into one of the most interesting microorganisms in human pathology. In developing countries *H. pylori* can affect up to 90% of the population and its treatment is based on antibiotic therapy, but this has the disadvantages of being
expensive, risks poor compliance, causes side effects and in particular, encouraging the emergence of resistance (Gold et al., 2000).

Currently, industrial probiotic foods are mainly dairy products, which have a microbial content in excess of $10^6$ CFU·cm$^{-3}$ at the end of their shelf life (Ouwehand et al., 1999). However, lactose intolerance and the cholesterol content are two drawbacks related to their consumption. Techniques such as vacuum impregnation though, make it possible to obtain fruit and vegetables enriched with probiotic microorganisms or minerals (Betoret et al., 2003). Vacuum impregnation allows, by means of pressure gradients, the incorporation of components into the structural matrix of foodstuffs without substantially modifying its organoleptic properties (Patent P99 02730-5 titled “Procedimiento de impregnación de alimentos naturales con productos nutracéuticos y dispositivo para su puesta en práctica”). The pressure gradients created in this system and the capillary pressure at the entrance of the pores produces an important transference of gas and liquid between the solid and the impregnating liquid (Fito et al., 2001; Betoret et al., 2003; Cháfer et al., 2003; Alzamora et al., 2005). Following vacuum impregnation, fruit and vegetables are highly unstable due to their high water content, and therefore it is necessary to apply a preservation method to increase their shelf life.

The development of a functional food from fresh fruit or vegetables with high microorganism content is extremely interesting not only because it represents an opportunity within the functional food industry but also because it can be used to reduce the disadvantages and side effects of traditionally used treatments. In order to commercialise a functional product it is necessary to prove scientifically, using both in vitro and in vivo studies its beneficial effects (Regulation CE N° 1924/2006). In the case of *H. pylori* infections, these studies should be directed towards reducing infection (Hamilton-Miller, 2003). To determine levels of infection, the breath test using labelled urea has been used extensively in studies (Michetti et al., 1999; De Vrese and Schrenmeir, 2000; Sakamoto et al., 2001).

The aim of this research is to determine both adequate material and process conditions to develop a probiotic low humid apple snack with potential effect against infection caused by Helicobacter pylori.

2. MATERIALS AND METHODS

2.1 Bacterial cultures.

*Lactobacillus salivarius* spp. *salivarius* CECT 4063 and *Lactobacillus acidophilus* CECT 903 were obtained from the Spanish Type Culture Collection (CECT).

2.2 Food materials.

Apples (cv. *Granny Smith*) from a local market were used to be vacuum impregnated. Peeled apples were cut into disc-shaped samples (5 mm thick, with a 65 mm external diameter and 20 mm internal diameter) following their vertical axis. Three samples were obtained from each apple and utilised for vacuum impregnation. Two commercial fruit juices, mandarin Don Simon® and
pineapple/grape Don Simon®, were selected as the impregnation liquid in order to minimise the effects on the characteristics of the fresh fruit.

2.3 Impregnation liquid preparation.

Lyophilised cultures were recovered following growth on MRS broth for 24h at 37°C. The cultures were then transferred into mandarin and pineapple/grape juices where the pH of the juices had been adjusted by the addition of sodium bicarbonate pH 5, 5.5 and 6. Four millilitres MRS broth with microorganism content of $10^9$ CFU/ml were added to 1 L of juice. The inoculated juices were grown for 24 and 48 hours at 37°C resulting in 12 different growth media: 6 mandarin and 6 pineapple/grape juices. The mandarin juice inoculated with *L. salivarius* spp. salivarius at pH 6 and after 24 hours incubation was achieved with maximum microbial content and was selected as impregnation liquid. The values stated are the average of five replicates.

2.4 Physicochemical characterisation.

Total soluble solids were measured in Brix units with a refractometer (ABBE ATAGO, NAR T3, Japan) at 20°C and pH values were determined with a potentiometer (micropH CRISON, 2001). The density of the juices was determined with a liquid picnometer. Water activity was measured using a dewpoint hygrometer (DECAGON Aqualab CX-2, ± 0,003) and the water content was quantified by vacuum drying at 60°C until a constant weight was achieved (method 20.013 AOAC, 1980). The values stated are the average of three replicates.

2.5 Microbial content.

The microbial content was determined following growth in MRS agar mandarin juice and pineapple/grape juice with pH values of 5, 5.5, 6 were counted on double layer MRS agar following incubation for 24 hours at 37°C.

To determine the microbial content of the impregnated samples, 5 grams of sample were mixed and crushed with 45 ml of buffered peptone water, following $10^{-1}$ to $10^{-8}$ dilutions the lactobacilli were counted on double layer MRS agar after incubation for 24 hours at 37°C.

To determine the microbial content of the dried samples, these were rehydrated with mandarin juice (pH6) at a ratio of $50 \text{cm}^3 \text{liquid} \cdot \text{g}^{-1} \text{sample}$ at 25°C over 24 hours. Following rehydration, the microbial content was determined as for the impregnated samples. For the dried samples, the microbial content was estimated with the mass balance performed in the rehydration operation (Betoret et al., 2003). The values stated are the average of ten replicates.

2.6 Methodology to produce probiotic enriched dried apple.

In the Figure 1 it is shown the flow chart to produce probiotic enriched dried apple.

The vacuum impregnation experiments were performed on a pilot scale using equipment designed in the Institute of Food Engineering for Development of the Polytechnic University in Valencia (Spain) (Fito et al., 2001). A vacuum
pressure of 50 mbar was applied for 10 min and then atmospheric pressure was restored. The samples were left submerged in the impregnation liquid for a further 10 minutes. The values stated are the average of ten replicates.

Impregnated apple samples were dried for 24 hours using a pilot scale air dryer (Martín, 2002) at 30°C under a flow rate of 4 m/s.

2.7 Preliminary in vivo study.

A preliminary study was undertaken involving 5 children (3 girls and 2 boys, age 11 years) chronically infected with *H. pylori* and patients of the Gastroenterology Paediatric office in Hospital Universitario Dr. Peset in Valencia. The children's diagnosis was confirmed before commencing treatment by the breath test using labelled $^{13}$C urea (commercial test TAU-KIT®) (Michetti et al., 1999; De Vrese and Schrenmeir, 2000; Sakamoto et al., 2001). TAU-KIT is a breath test suitable for in vivo diagnosis of gastroduodenal *Helicobacter pylori* infection. Each soluble tablet contains 100 mg of $^{13}$C-urea and excipients. During the 28 day study, 30g of the dried product with a moisture content of 0.144 ± 0.012 g_{water·g^{-1}_{sample}} and microorganism content of (9.5 ± 0.2)·10^7 CFU·g^{-1}_{dried sample} was supplied daily to each child. At the end of the study the urea breath test was repeated. The study has been done following rigorous ethical standards. An ethical approval was obtained by the Hospital Universitario Dr. Peset and the Universidad Politécnica de Valencia to carry out the study.

2.8 Statistical analysis.

To determine the statistical significance of the results obtained, an analysis of variance test was carried out (ANOVA) with 95% confidence levels ($p \leq 0.05$) using the program STATGRAPHICS PLUS v.5.1.

3. RESULTS AND DISCUSSION

3.1 Use of mandarin and pineapple/grape juices as the impregnation liquid.

The use of vacuum impregnation to obtain fruit enriched with probiotic microorganisms requires that the impregnation liquid is able to enter the pores or intercellular spaces. However certain physicochemical properties of the impregnation liquid are required; the pH of the impregnation liquid and the fruit must be such to allow growth of the microorganism, the viscosity of the impregnation liquid should also allow flux inside the pores or intercellular spaces (Martínez-Monzó et al., 1998) and as much as possible the natural characteristics of the impregnated fruit should not be affected. Furthermore, the concentration of microorganisms in the impregnation liquid must be sufficiently high enough to obtain adequate levels of microorganisms in the impregnated fruit. Some physicochemical properties of commercial mandarin and pineapple/grape juice were determined and the results are shown in Table 1. Martinez-Monzó (1998) studied pectin dissolutions and determined viscosities below 10mPa.s do not affect impregnation procedure. In our case, concentration and composition of impregnation liquid ensure not reach this value of viscosity at any time of the process.
The growth media was prepared as described and inoculated with strains of the microorganisms under consideration. The cultures were incubated for 24 or 48 hours to obtain the maximum possible concentration of microorganisms. The final microbial content for each medium is shown in Table 2.

A multiple analysis of variance (95% confidence levels) showed that the different juices, pH and incubation periods have statistically significant effects on the microbial content. In addition, there were no double interactions in the model, suggesting that the effect a single factor has on the microbial content is independent of the other factors. From this analysis *L. salivarius* growth, results in higher microbial content than *L. acidophilus* under the experimental conditions tested. Furthermore, as shown in table 2, the highest microbial content ($10^8$ CFU·cm$^{-3}$) was obtained with *L. salivarius*, cultured in mandarin juice at pH 5.96, following incubation for 24 hours. Therefore, this growth medium was selected as the impregnation liquid.

In order to analyze any changes that the growth of the microorganism produced in the mandarin juice which had been selected as the impregnation liquid, some physicochemical parameters were determined (Table 1). It was noted that the sugar content of this inoculated juice was slightly below that of the commercial juice. This was thought to be due to the growing microorganism utilising some of the available sugars. The inoculated mandarin juice was slightly denser than fresh mandarin juice, although these differences were minimal.

3.2 Physicochemical characterisation and microbial content in impregnated samples.

The physicochemical characteristics (pH, water activity, moisture content) and microorganism content determined in the samples at each stage of the process are shown in Table 3.

The water activity value of mandarin juice (Table 3) was similar to that of the apple that was to be impregnated (Table 3). Therefore, it was expected that during the vacuum impregnation, the only transfer mechanism would be hydrodynamic (Fito et al., 1996) and the characteristics of the fresh apple would not be highly altered (Table 3).

In vacuum impregnation experiments performed using mandarin juice with a microbial content as in Table 3, following the theory of the hydrodynamic model proposed by Fito, 1994; Fito and Pastor in 1994 and Fito et al., 1996 it was possible to calculate the values of volumetric deformation ($\gamma = -0.14 \pm 0.07$ cm$^3$·cm$^{-3}$ sample), effective porosity ($e_o = 0.201 \pm 0.012$ cm$^3$·cm$^{-3}$ sample) and the volumetric impregnation levels reached ($X = 0.1954 \pm 0.0106$ cm$^3$·cm$^{-3}$ sample) in the samples. These values are similar to those obtained in previous studies with apple juice (Betoret et al., 2003).

Taking into account the pore size of the apple (6.64 ± 0.24 μm) (Bazhal et al., 2003) and the size of the individual *Lactobacillus* cells (0.7x1.6 μm) (Zapata et al., 2009) a homogeneous impregnation was achieved. Additionally, it was possible to estimate theoretically the microbial content of the impregnated samples (Equation 1).
Where:

$m$; mass (g)

$x$; microorganism content (CFU/g or CFU/ml)

$X$; volumetric impregnation (cm$^3$/cm$^3_{sample}$)

$\rho$; density (g/cm$^3$)

$fIV$; sub index referred to impregnated fruit

$ff$; sub index referred to fresh fruit

$LIV$; sub index referred to impregnation liquid

$I$; sub index referred to impregnation operation

The estimated microbial content in the apple following vacuum impregnation was $(1.04 \pm 0.08) \cdot 10^8$ CFU·g$^{-1}$. This calculated value is similar to that obtained experimentally $(1.51 \pm 0.07) \cdot 10^8$ CFU·g$^{-1}$ impregnated sample and thus confirms that the impregnation operation was homogeneous and the principal transfer mechanism was hydrodynamic.

The high values of water activity in the impregnated samples (Table 3) made them highly unstable. Following hot air drying, the resulting low value obtained for water activity (Table 4) does not permit the growth of fungi or other harmful microorganisms and dried samples are stable without losing their probiotic effect for at least two weeks when stored at room temperature (Betoret et al., 2003).

3.3 Microbial determination in the dried samples.

In order to determine the microbial content of the dried samples and due to their low humidity content, those must be rehydrated. Following the flow chart showed in the figure 2, the microbial content of the rehydrated samples and the microbial content of the residual rehydration liquid were required.

In the case of a system consisting in a impregnated apple immersed in a sterile liquid medium and subjected to the rehydration operation, the general microorganism content balance equation is:

$\sum x_s \cdot m_s - \sum x_e \cdot m_e + \left[ \frac{M \cdot dx}{dt} \right] = G_j \hspace{1cm} (Eq. 2)$

Where:

$m$; mass flow (g/s)

$M$; Total mass (g)

$x$; microorganism content (CFU/g)

$G_j$; generated microorganism content (CFU/s)

$s$; sub index referred to flow outputting

$e$; sub index referred to flow inputting

Assuming as hypothesis the total mass in the system does not vary with time.

The equation 2 stays:

$\sum x_s \cdot m_s = \sum x_e \cdot m_e + G_j \hspace{1cm} (Eq.3)$

If applied to the rehydration operation showed in the Figure 2:

$m_{df} \cdot x_{df} + m_{trh} \cdot x_{trh} = m_{fr} \cdot x_{fr} + m_{tr} \cdot x_{tr} + G_j \hspace{1cm} (Eq.4)$

Where sub index:
df; dry fruit
lrh; rehydration liquid
fr; rehydrated fruit
lr; residual rehydration liquid

Taking into account that the microbial concentration in rehydration liquid is zero, the microbial content of dry sample can be calculated as follows:

\[
x_{df} = \frac{m_{fr} \cdot x_{fr} + m_{lr} \cdot x_{lr} - m_{lrh} \cdot x_{lrh} + G_i}{m_{df}} \quad (Eq. 5)
\]

We consider there is no generation of microorganism during rehydration. In fact, means that the calculated microbial content is the minimum possible.

Table 4 shows the physicochemical characteristics (water activity, moisture content) and microorganism content determined in the dry samples.

As it is possible to see, the microbial content of the rehydrated samples was slightly less than that of the impregnated samples prior to drying (Table 4). This reduction in microbial content could be explained by a possible transfer of \( L. \) \( \text{salivarius spp. salivarius} \) from the surface or near pores of apple to the rehydration liquid. To compare the microbial content of the impregnated and the dried samples, the microbial content of both was expressed per gram of dry matter, resulting in microbial content of impregnated samples \( (1.51 \pm 0.07) \cdot 10^8 \) CFU\( \cdot g^{-1} \) dry matter and microbial content of dry samples \( (9.5 \pm 0.2) \cdot 10^7 \) CFU\( \cdot g^{-1} \) dry matter.

Air drying of the impregnated samples resulted in a 1-log fold reduction in microbial content. Despite the observed reduction, the final product had a high microbial content as the viable count of \( L. \) \( \text{salivarius spp. salivarius} \) was very close to \( 10^8 \) CFU\( \cdot g^{-1} \). This new product manufactured from apple and mandarin juice achieved the \( 10^7 \) CFU\( \cdot g^{-1} \) of sample, this value corresponds to average values found in commercial probiotic dairy products and according to the literature would be sufficient to be potentially effective against \( H. \) \( \text{pylori} \) infections (Cruchet et al., 2003; De Champs et al., 2003).

3.4 Preliminary in vivo test.

The \( ^{13} \)C urea breath test was undertaken by all participants at the start and end of the study. After oral ingestion, the urea reaches the gastric flora. In the presence of \( H. \) \( \text{pylori} \), the \( ^{13} \)C-urea is metabolized by the enzyme urease:

\[
2H_2N\left(^{13}\text{CO}\right)NH_2 + 2H_2O \rightarrow 4\text{NH}_3 + 2^{13}\text{CO}_2
\]

Urease is produced in the stomach only by \( H. \) \( \text{pylori} \). Other urease-producing bacteria rarely have been found in the gastric flora. Carbon dioxide diffuses into the blood vessels. From there it is transported as bicarbonate to the lungs and released as \( ^{13} \)CO\(_2\) in expired air. In the presence of bacterial urease, the ratio of carbon isotopes \( ^{13}\text{C}/^{12}\text{C} \) change significantly. The proportion of \( ^{13} \)CO\(_2\) in exhaled air samples was determined by mass spectrometry isotope ratio (IRMS) and set as absolute difference (value \( \Delta \delta \)) between the values at 0 and 30 minutes. The critical point to determine, \( H. \) \( \text{pylori} \) negative or positive is the value of \( \Delta \delta \) 4 % to 5 %, which means that an increase in value over \( \Delta \delta \) 4 % to 5 % indicates that there is infection. In the absence of bacterial urease, all administered urea, after absorption in the gastrointestinal tract, is metabolized as endogenous urea.

During the 28 day study, each child daily received 30 g of the dried product with a moisture content of \( 0.144 \pm 0.012 \) g\( \cdot \) water\( \cdot \) sample and a microorganism
content of \((2.850 \pm 0.003) \times 10^9\) CFU·day\(^{-1}\). According to the literature, the consumption of \(10^9\) CFU per day of *L. salivarius* spp. *salivarius* for four weeks is sufficient to have an effect on *H. pylori* infection (Aiba et al., 1998). In all patients was possible to detect a decrease in the values of \(^{13}\text{C}\) for the basal sample in the expired breath. Four of the five children who took part in the study completed the 28 days treatment; whilst one child (with values from 6 ppm at the start to 5 ppm at the end of the study) ate the product only for the first 15 days. Two children (one with values from 5 to 0.3 ppm and the other with values from 6 to 4 ppm) reported improvements in their previous symptoms, which consisted of abdominal problems and aerophagia. Although five patients are not enough to establish significance, the results obtained suggest an improvement in the symptoms caused by the infection and therefore it is necessary to carry out further studies involving more patients to properly evaluate the effectiveness of the food. During the study, the children were asked by qualified personnel about the appearance, flavour and consistency of the product. While the flavour was acceptable, both the appearance and consistency of product were not liked and all would have preferred to eat a crisper product.

### 4. CONCLUSIONS

The nutritional and physicochemical properties of mandarin juice (with the pH adjusted to 6) resulted in \(10^8\) CFU·cm\(^{-3}\) of *L. salivarius* spp. *salivarius*, following 24 hours incubation, allowing this solution to be used as the liquid medium for vacuum impregnation of samples of fresh apple.

The impregnation parameters obtained and the microorganism content achieved confirmed the validity of this approach to the development of a natural low moisture content food with a probiotic effect.

Further studies involving more patients are needed to properly evaluate the effectiveness of this food; however the *L. salivarius* spp. *salivarius* microbial content in the final product and the results of the *in vivo* pilot test confirm the potentially beneficial effects against *H. pylori* infection.

### 5. REFERENCES


Patent P99 02730-5 titled “Procedimiento de impregnación de alimentos naturales con productos nutracéuticos y dispositivo para su puesta en práctica”

Reglamento (CE) Nº 1924/2006 del parlamento europeo y del consejo de 20 de diciembre de 2006 relativo a las declaraciones nutricionales y de propiedades saludables en los alimentos.


