Betoret Valls, ME.; Betoret Valls, N.; Arilla, A.; Bennár ., M.; Barrera Puigdollers, C.; Codoner, P.; Fito Maupoey, P. (2012). No invasive methodology to produce a probiotic low humid apple snack with potential effect against Helicobacter pylori. Journal of Food Engineering. 110(2):289-293. doi:10.1016/j.jfoodeng.2011.04.027.

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

E. Betoret[a], N. Betoret[a], A. Arilla[a], M. Bennár[a], C. Barrera[a], P. Codoñer[b] and P. Fito*[a]

a Institute of Food Engineering for Development, Department of Food Technology, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain

b Department of Pediatrics, Obstetrics, and Gynecology. Facultad de Medicina y Odontología, Universidad de Valencia, Avenida Blasco Ibáñez, 15, 46010 Valencia (Spain)

* Corresponding author. Address: Institute of Food Engineering for Development, Department of Food Technology, Polytechnic University of Valencia, Camino de Vera s/n, 46022 Valencia, Spain. Tel.: +34 96 387 7367. Fax.: +34 96 387 7056.
E-mail address: pfito@tal.upv.es

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 $g_{water} \cdot g^{-1}_{sample}$) ensured stability, and although the drying process affected the microbial content, the concentration in the final product (9.486 ± 0.013)·10^7 $CFU \cdot g^{-1}_{dry\ sample}$) 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.

Keywords: *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 (10^9–10^{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 Nº 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 37º 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 10^9CFU/ml were added to 1 L of juice. The inoculated juices were grown for 24 and 48 hours at 37ºC 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 20ºC 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 (DECAGÓN Aqualab CX-2, ± 0,003) and the water content was quantified by vacuum drying at 60ºC 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 37ºC.

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 37ºC.

To determine the microbial content of the dried samples, these were rehydrated with mandarin juice (pH6) at a ratio of 50 $cm^3_{liquid} \cdot g^{-1}_{sample}$ at 25ºC 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 30ºC 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 $g_{water} \cdot g^{-1}_{sample}$ and microorganism content of $(9.5 \pm 0.2) \cdot 10^7$ $CFU \cdot g^{-1}_{dried\ sample}$ 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 (γ = -0.14 ± 0.07 $cm^3 \cdot cm^{-3}_{sample}$), effective porosity ($\varepsilon_o$ = 0.201 ± 0.012 $cm^3 \cdot cm^{-3}_{sample}$) and the volumetric impregnation levels reached (X = 0.1954 ± 0.0106 $cm^3 \cdot cm^{-3}_{sample}$) 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).

$$x_{fIV} = \frac{x_{LIV} \cdot X \cdot \frac{\rho_{LIV}}{\rho_{ff}}}{1 + X \cdot \frac{\rho_{LIV}}{\rho_{ff}}} \ (Eq.1)$$

Where:

m; mass (g)

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

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

ρ; 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

IV; 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:

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

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:

$$\sum x_s \cdot m_s = \sum x_e \cdot m_e + G_j \ (Eq.3)$$

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

$$m_{df} \cdot x_{df} + m_{lrh} \cdot x_{lrh} = m_{fr} \cdot x_{fr} + m_{lr} \cdot x_{lr} + G_j \ (Eq.4)$$

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:

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

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. 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, then the microbial content of both was expressed per gram of dry matter, resulting on microbial content of impregnated samples (1.51 ± 0.07)·10^8 CFU·$g^{-1}_{\text{dry matter}}$ and microbial content of dry samples (9.5 ± 0.2)·10^7 CFU·$g^{-1}_{\text{dry matter}}$

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. salivarius spp. salivarius* was very close to 10^8 CFU·g^{-1}, This new product manufactured from apple and mandarin juice achieved the 10^7CFU·$g^{-1}_{\text{ of sample}}$, 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. 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 mucosa. In the presence of *H. pylori*, the ^{13}C-urea is metabolized by the enzyme urease:

$$2H_2N(^{13}CO)NH_2 + 2H_2O \rightarrow 4NH_3 + 2^{13}CO_2$$

Urease is produced in the stomach only by H. 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 $^{13}C/^{12}C$ 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 Δδ) between the values at 0 and 30 minutes. The critical point to determine, *H. pylori* negative or positive is the value of Δδ 4 ‰ to 5 ‰, which means that an increase in value over Δδ 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 ± 0.012 $g_{water}·g^{-1}_{sample}$ and a microorganism

content of (2.850 ± 0.003)·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}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

A.O.A.C., (1980). Association of official analytical chemist. Official Methods of Analysis, 20013. Washington, D.C.

Aiba Y., Suzuki N., Kabir A.M., Takagi A., Koga, Y. (1998). Lactic acid-mediated suppression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. *American Journal of Gastroenterology*, 93, 2097-2101.

Alzamora S.M., Salvatori D., Tapia M.S., López-Malo A., Welti-Chames J., Fito P. (2005). Novel functional foods from vegetable matrices impregnated with biologically active compounds. *Journal of Food Engineering*, 67, 205-214.

Bazhal M.I., Ngadi M.O. (2003). Influence of pulsed electroplasmolysis on the porous structure of apple tissue. *Biosystems Engineering*, 86, 51-57.

Betoret N., Puente L., Díaz M.J., Pagán M.J., García M.J., Gras M.L., Martínez-Monzó J., Fito P. (2003). Development of probiotic-enriched dried fruits by vacuum impregnation. *Journal of Food Engineering*, 56, 273-277.

Canducci F., Armuzzi A., Cremonini F., Cammarota G., Bartolozzi F., Pola P., Gasbarrini G., Gasbarrini A. (2000). A lyophilized and inactivated culture of Lactobacillus acidophilus increases *Helicobacter pylori* eradication rates. *Alimentary Pharmacology & Therapeutics*, 14, 1625-9.

Cháfer M., González-Martínez C., Fernández B., Pérez L., Chiralt, A. (2003). Effect of Blanching and Vacuum Pulse Application on Osmotic Dehydration of Pear. *Food Science and Technology International*, 9, 321-328.

Cruchet S., Obregon M.C., Salazar G., Diaz E., Gotteland M., (2003). Effect of the ingestion of a dietary product containing *Lactobacillus johnsonii La1* on *Helicobacter pylori* colonization in children. *Nutrition,* 19, 716-721.

De Champs C., Maroncle N., Balestrino D., Rich C., Forestier C. (2003). Persistence of colonization of intestinal mucosa by a probiotic strain*, Lactobacillus casei subsp. Rhamnosus Lcr35,* after oral consumption. *Journal of Clinical Microbiology,* 41, 3, 1270-1273.

De Vrese M., Schrezenmeir J. (2000). Einfluss probiotischer Bifidobakterien auf die *Helicobacter pylori* activitat. *Federal Dairy Center, Annual report*.

Fito P. (1994). Modelling of vacuum osmotic dehydration of food. *Journal of Food Engineering,* 22, 1-4, 313-328.

Fito P., Andrés A., Chiralt A., Pardo P. (1996). Coupling of hydrodinamic mechanism and deformation relaxation phenomena during vaccuum treatments in solid porous food-liquid systems. *Journal of Food Engineering*, 27, 229-240.

Fito P., Chiralt A., Betoret N., Gras M.L., Cháfer M., Martínez-Monzó J., Andrés A., Vidal D. (2001). Vacuum impregnation and osmotic deshydratation in matrix engineering. Application in functional fresh food development. Journal of Food Engineering, 49, 175-183.

Fito P., Pastor R. (1994). On some non-difusional mechanism occurring during vacuum osmotic dehydration. *Journal of Food Engineering*, 21, 513-519.

Fuller R. (1989). Probiotics in man and animals. *Journal of Applied bacteriology*, 66, 365–378.

Gold B.D., Colletti R.B., Abbott M., Czinn S.J., Elitsur Y., Hassall E., Macarthur C., Snyder J., Sherman P.M. (2000). *Helicobacter pylori* Infection in Children: Recommendations for Diagnosis and Treatment. *Journal of Pediatric Gastroenterology and Nutrition,* 31, 490–497.

Hamilton-Miller J.M.T. (2003). The role of probiotics in the treatment and prevention of *Helicobacter pylori* infection. *International Journal of Antimicrobial Agents*, 22, 360-366.

Martin, E. (2002). Utilización de microondas en el secado por aire caliente de manzana (var. Granny Smith). Influencia del pretratamiento por impregnación a vacío. Universidad Politécnica de Valencia. Tesis doctoral.

Martínez-Monzó J., Martínez-Navarrete N., Chiralt A., Fito P. (1998). Mechanical and structural changes in apple (var. Granny Smith) due to vacuum impregnation with cryoprotectants. *Journal of Food Science*, 63, 499-503.

Michetti P., Dorta G., Wiesel P.H., Brassart D., Verdu E., Herranz M., Felley C., Porta N., Rouvet M., Blum A.L., Corthésy-Theulaz I. (1999). Effect of whey-based culture supernatant of *Lactobacillus acidophilus (johnsonii) La1* on *Helicobacter pylori* infection in humans. *Digestion*, 60(3), 203-9.

Mrda Z., Zivanovic M., Rasic J., Gajin S., Somer L., Trbojevic S. (1998). Therapy of Helicobacter pylori infection using *Lactobacillus acidophilus*. *Meditsinski Pregled*, 51, 343-345.

Ouwehand, A., Kirjavainen, P., Shortt, C., Salminen, S. (1999). Probiotics: mechanisms and stabilised effect. *International Dairy. Journal*, 9, 43–52.

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.

Sakamoto I., Igarashi M., Kimura K., Takagi A., Miwa T., Koga Y. (2001). Suppressive effect of *Lactobacillus gasseri* OLL 2716 (LG21) on *Helicobacter pylori* infection in humans. *Journal of Antimicrobial Chemotherapy,* 47, 5, 709-710.

Salminen S., Ouwehand A., Benno Y., Lee Y.K. (1999). Probiotics: how should they be defined?. *Trends in Food Science & Technology*, 10, 3, 107-110.

Sanders M. E. (1999). Probiotics. *Food Technology*, 53, 67–77.

Sheu B.S., Wu J.J., Lo C.Y., Wu H.W., Chen J.H., Lin Y.S., Lin M.D. (2002). Impact of supplement with *Lactobacillus*- and *Bifidobacterium*-containing yogurt on triple therapy for *Helicobacter pylori* eradication. *Alimentary Pharmacology & Therapeutics*, 16, 9, 1669–1675.

Zapata S., Muñoz J., Ruiz O.S., Montoya O.I., Gutiérrez P.A. (2009). Isolation of *Lactobacillus plantarum* LPBM10 and partial characterization of its bacteriocin. *Revista de la facultad de química farmacéutica,* 16, 1, 75-82.