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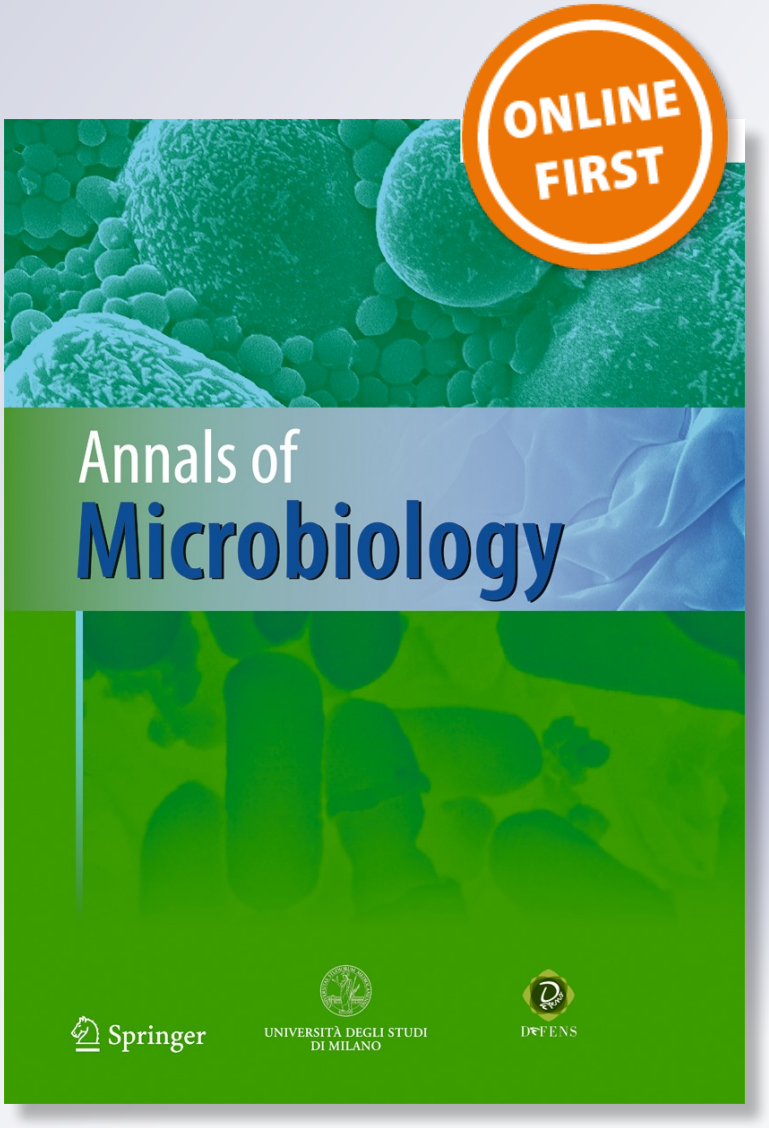

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ORIGINAL ARTICLE

Apple juice as a medium for fermentation by the probiotic *Lactobacillus plantarum* PCS 26 strain

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Abstract Studies on the development of non-dairy probiotic foods and beverages are continuously emerging. Fruit and vegetable juices have proved to be promising carriers or growth media for probiotics. In this study, apple juice was explored as a growth medium for cultivation of the probiotic *Lactobacillus plantarum* PCS 26 strain. Fermentation was performed with free and Ca-alginate–embedded bacteria at different initial pH values, as well as with whey supplementation as a growth enhancer. During the fermentation, growth kinetics, pH, sugars consumption and lactic acid production were measured, along with culture survival during storage. The best results were achieved by fermentation at an initial pH of 4.2, reaching maximal cell density of 2.5 x 10^9 CFU/mL and a final pH of 4.7 after 24 h. Malolactic conversion was commenced by the strain as energy yielding mechanism, thereby lowering the consumption of sugars below the limit of determination by the analytical method used. Apple juice supplementation with 5 %v/v whey accelerated fermentation kinetics and resulted in a higher viable bacterial count. In contrast, entrapment of cells into Ca-alginate caused significantly slower growth, yielding a lower bacterial count at the end of fermentation (3.2 x 10^6 CFU/mL). However, stability during storage of the fermented product at 4–7 °C improved, and the survival of immobilized bacteria, estimated by Weibullian model, increased to 32.1±5.2 days compared to 22.0±0.68 days in the free-cell fermentation. In conclusion, apple juice was found to be an appropriate medium for fermentation by probiotic *Lactobacillus plantarum* PCS 26, resulting in a functional drink with potentially good sensory acceptance and shelf life.

Keywords *Lactobacillus plantarum* PCS 26 · Probiotic · Apple juice · Malolactic fermentation · Cell immobilization

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Introduction

In recent years, a growing public awareness about diet-related health issues has significantly increased scientific and commercial attention on microorganisms with beneficial effects on human health (Granato et al. 2010), commonly known as probiotics. There are plenty of probiotic bacterial strains available with specific health benefits; these are usually offered in the market in the form of fermented dairy products. Ideally, in terms of costs and technology, probiotic bacteria should be capable of fermenting products by themselves. However, in most industrial technological practices, fermentations are performed separately, and ready-to-use probiotic cultures are added into the final product. Survival of these bacteria in the final product during shelf life and during digestion is an important consideration for proper selection of strains for food applications.

Lactose intolerance and cholesterol content are the two major drawbacks related to probiotic foods based on dairy products (Betoret et al. 2012). In addition, the demand for vegetarian probiotic products, in particular in the developed countries, is irising with the increasing number of vegetarian consumers. Therefore, developing new products of non-diary origin enriched with probiotics is considered to be a promising field of work (Bernat et al. 2014a). Despite the existence of traditional drinks from Africa and the Far East countries made

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by spontaneous fermentations of cereals with native lactic acid bacteria, commercial products starting with cereals (Proviva – first non-dairy product, Skane Dairy, Sweden) to aromatic plants and fruit-based products have been already manufactured with defined probiotics (Prado et al. 2008). Published data dealing with the fermentation of juices from carrots (Sharma and Mishra 2013; Tamminen et al. 2013), beet root (Czyżowska et al. 2006), cabbage (Yoon et al. 2006), tomato (Yoon et al. 2004), cucumber (Buruleanu et al. 2012) and their mixtures, as well as banana (Tsen et al. 2004), orange (Sheehan et al. 2007) and other tropical fruits (Saw et al. 2011), are found in literature.

The nature of the food carrier is also important for the survival of bacterial strains during their passage through the gastro-intestinal tract (Goyal and Gandhi 2008). Researchers found that the protein from peanut flour fermented by *Lactobacillus plantarum* P9 can enhance the survival of probiotic cells in simulated gastric and bile juices (Wang et al. 2007). Physical protection of probiotics by microencapsulation is one of the most-used approaches for improvement of probiotic survival during storage and after consumption (Rokka and Rantamäki 2010; Todorov et al. 2012). The most common methods for microencapsulation of probiotics include encapsulation by extrusion, emulsification, spray drying and freeze drying, while alginate, chitin, pectin, k-carrageenan are the most commonly used carriers (Tsen et al. 2004; Özer et al. 2009). Immobilization of probiotics for the manufacture of beverages is commonly used by many researchers to achieve high cell density fermentations for both cell and metabolite production. The size of the microcapsules is important, because it influences the mass transfer between the cell and the surrounding medium, as well as the sensory properties of foods. Namely, large gel beads can deteriorate mouth-feel properties of a fluid product. The diameters of the resulting gel beads formed by the extrusion technique are determined by the nozzle diameter and fluid viscosity (Kailasapathy 2002), and can vary from 1.5 mm (Krasaekoopt et al. 2007) to 3 mm (Tsen et al. 2004). Microencapsulation by emulsification and spray drying produces beads with much smaller diameter (0.1–1.0 mm), but these techniques are more expensive than the simple extrusion method.

Whey was also found to protect the ingested probiotic cells by increasing the overall pH in the gut and by inhibiting the activity of digestive enzymes (Charteris et al. 1998). Whey contains proteins with very high biological value and it is a good source of electrolytes and minerals. According to Naidu et al. (1999), sialic acid is a component of whey that possesses prebiotic activity. As whey is rich in nutrients and growth factors that have potential to stimulate the growth of lactic acid bacteria, probiotic beverages using this milk by-product (Hernandez-Mendoza et al. 2007) or mixtures of it and fruit juices (Dalev et al. 2006; Shukla et al. 2013) have been developed. However, as lactose is the most abundant component in whey, reaching 70 % of the total solids, it cannot be consumed in very high concentrations by lactose intolerant people.

The objective of this study was to determine the suitability of a commercial apple juice as a medium for production of a probiotic beverage using the probiotic bacterium *L. plantarum* strain PCS 26, isolated from traditional Slovenian cheese (Nissen et al. 2009). The strain was recently studied extensively for its effects on the human digestive tract, and the results were very promising (Dimitrovski et al. 2014). In the current study, the technological properties of *L. plantarum* PCS 26 for production of probiotic beverage were examined. The effects of initial fermentation conditions, additions of whey as a nutrient supplement and the encapsulation of bacteria into Ca-alginate were considered for increasing the cell density and survivability of the bacterial strain in the fermented apple juice during cold storage.

Materials and methods

Bacterial strains and culture conditions

Probiotic *L. plantarum* PCS 26 (Deposited at Microbial Strain Collection of Latvia, accession number: PCS 26 (P 975)) was isolated from Slovenian traditional cheese (PathogenCombat 2011). Cryopreserved culture was kept at −20 °C and revitalized by overnight growth in de Man, Rogosa and Sharpe (MRS) broth (Merck, Whitehouse station, New Jersey, USA) at 37 °C, using semi-anaerobic conditions.

Cell immobilization

An overnight culture of *L. plantarum* PCS 26 was washed twice with peptone water (0.1 %w/v). Cell suspension (100 μL) was then mixed with 10 mL of 2 %w/v Na-alginate (Sigma-Aldrich, St. Louis, Missouri, USA) and extruded through a needle, using a syringe to form droplets. The droplets were dropped into a gently stirred 0.1 M $CaCl_2$ (Merck) solution and kept in the solution for 2 h to obtain Ca-alginate gel beads with entrapped cells. The immobilization efficiency (η) was calculated as a ratio between immobilized cells and the total number of cells (immobilized plus free) (Velickova et al. 2009).

Supplementation of apple juice with whey

Pasteurized whey in liquid form was obtained from a local dairy production facility and was aseptically added into the apple juice. For testing the effect of whey on the growth of the culture, apple juice was supplemented with 5, 10 or 15 %v/v whey, yielding apple juice/whey ratios of 95/5, 90/10 and 85/15 v/v.

Inoculation and fermentation

Commercial apple juice (100 %, no sugars added, no preservatives) was obtained from the local supermarket and used as a medium for fermentation. The original pH value of the juice was 3.5, as measured by a pH-meter (Sartorius PB-11, Goettingen, Germany). The adjustment of the pH to higher values of 4.2 and 5.1 was done by the addition of 3.3 and 6.7 g/L of Na_2CO_3, respectively. An overnight culture of *L. plantarum* PCS 26 incubated on MRS broth at 37 °C was used as inoculum. The absence of microorganisms in the apple juice prior to inoculations was confirmed by total plate count. Batch fermentations were carried out with 300 mL of apple juice placed in a 500-mL Erlenmeyer flask on a rotary shaker (120 rpm) at 37 °C for 48 h. The total fermentation time of 48 h is not presented in all graphs, because the data points at the end of the fermentation did not contain any additional information about the fermentation. Inoculation with 30 μL of overnight culture was enough to obtain an initial viable count of about 10^7 CFU/mL in all fermentations. Samples were taken aseptically at appropriate time intervals.

Bacterial viable count

Bacterial viable count, marked as N (CFU/mL), was measured by the plate count technique. After a series of appropriate dilutions, the samples were planted on MRS agar plates and incubated at 37 °C for 48 h. The grown colonies were manually counted, and this number was multiplied by the plate dilution, resulting in a bacterial count; colony forming units per mL of apple juice (CFU/mL). For immobilized cells, ten Ca-alginate beads were gently shaken in 0.28 M KH_2PO_4 solution (Merck) for 15 min at room temperature for depolymerization of the beads and release of the immobilized cells (Zerajic et al. 1990). The number of colonies obtained from these ten beads was used to calculate the number of viable entrapped bacteria in the medium (CFU/mL). The total number of beads was calculated by measuring the weight of 30 beads and then comparing it with the weight of all beads.

Determination of sugar and acid concentration

High Performance Liquid Chromatography (HPLC, Agilent Technologies, Inc., Santa Clara, United States) was used for quantification of sugar concentration in the samples during the fermentation. A Supelcosil LC-NH_2 column, 250×4.6 mm, 5 μm particle size (Supelco analytical, Sigma Aldrich Group, Taufkirchen, Germany) separated the containing sugars in 15 min using isocratic mobile phase acetonitrile/water=75/25 %v/v at 40 °C (Muntean and Muntean 2010). A refractive index detector, also thermostated at 40 °C, was used for detection of the analytes, and the data were processed by Agilent ChemStation software.

The concentration of acids was determined using a Shimadzu Prominence liquid chromatography system (Shimadzu corp., Kyoto, Japan). Separation of the compounds was achieved at 55 °C using Aminex HPX-87H column, 300 mm×7.8 mm ID, 5 μm particle size (Bio-Rad Laboratories, California, United States), by an in-house method. Isocratic elution was applied with a mobile phase consisting of 2.5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The wavelength selected for detection purposes was 214 nm and the measured data were processed by Class VP 7.3 software.

Concentrations of the measured components were quantified from the peak areas by using appropriate standard curves generated with external standards.

Preliminary sensory evaluation

Preliminary sensory analysis of the fermented apple juice was carried out in a standardised test room. Four samples were tested: apple juice with and without supplementation with 5 %v/v whey produced by free-cell fermentation initiated at pH 4.2; apple juice produced by free-cell fermentation initiated at pH 5.1 and apple juice fermented with immobilized cells. The samples of fermented apple juices were served in transparent plastic cups labelled with three-digit random numbers. Commercial apple juice was used as a referent sample. General acceptance of smell, color, transparency and taste of the juices was evaluated by three panelists with no prior knowledge of the experiments. Unacceptable, acceptable and very good were used as attributes of the hedonic quality of the fermented apple juices.

Lactobacillus plantarum PCS 26 stability during storage

The fermented apple juice at the time point of maximal cell concentration was refrigerated at low temperature (4–7 °C), and samples for viable count measurement were taken at regular intervals during the following 17 days.

Statistical analysis and kinetic modeling

The presented growth curves are representative results of several fermentations where the data are average values of triplicate measurements of the samples. The presented data for the HPLC analysis are average values of three independent fermentations. Standard deviations are shown as error bars.

For a description of microbial kinetics, three static growth models were fitted to the colony count data: the shifted logistic function, th emodified logistic function and the modified Gompertz model (Eqs. 1–3) (van Boekel 2009). The goodness of fit was determined by the Akaike Criterion and the model

that best represented the data was used to estimate the kinetic parameters.

$$Y(t) = Y_{A_s}\left[\frac{1}{1+\exp(k(t_c-t))} - \frac{1}{1+\exp(kt_c)}\right] \quad (1)$$

with Y_{As} loosely interpreted as asymptotic value (i.e., ln N_{max}/N_o), k related to specific growth rate and t_c is the time of the lag phase.

$$ln\frac{N}{N_o} = \frac{A_s}{1+\exp\left[\frac{4\mu_{max}}{A_s}(\lambda - t) + 2\right]} \quad (2)$$

$$ln\frac{N}{N_o} = A_s exp\left[-exp\left(\frac{\mu_{max}e}{A_s}(\lambda - t) + 1\right)\right] \quad (3)$$

In these two equations, the parameters are re-parameterized in the microbial interpretable parameters: N *and* N_o represent the colony count at time t and at t=0, respectively; *As* is asymptote (max growth cycles ln N_{max}/N_o); μ_{max} is maximal specific growth rate; and λ is the time of the lag phase.

A nonlinear survival model, the Weibull distribution function (Eq. 4) was fitted to the data derived from the strain survival during storage, and was used to estimate the specific survival rate (van Boekel 2009). Hence, the storage time by which the probiotic juice would retain viable count above 10^6 CFU/mL was predicted.

$$log\frac{N}{No} = -b \cdot t^n \quad (4)$$

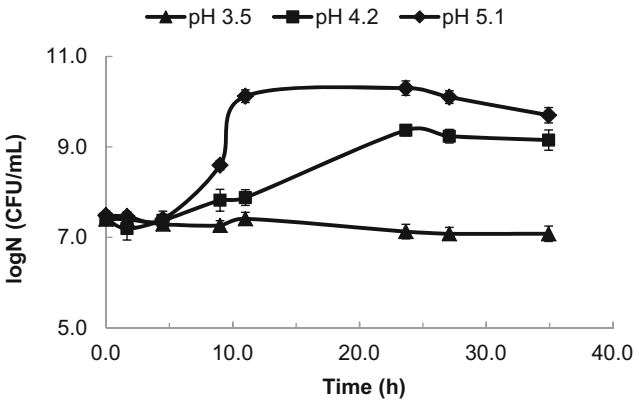
With $b = \frac{1}{2.303} \cdot \left(\frac{1}{\alpha_w}\right)^{\beta_w}$ and $n=\beta_w$ where α_w and β_w are the two parameters of the distributions; α_w is a scale parameter (a characteristic time) and the β_w is the so-called shape parameter.

Results and discussion

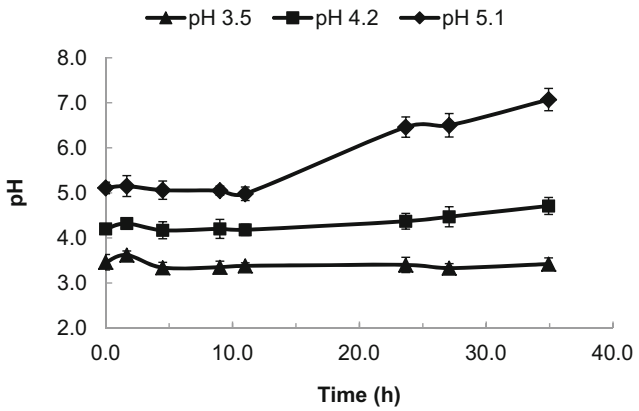
Fermentation with free *L. plantarum* PCS 26 cells

Effect of initial pH value

The results from the fermentation of apple juice by *L. plantarum* PCS 26 performed at three initial pH values, 3.5, 4.2 and 5.1, are presented in Fig. 1. In all three cases, the fermentation started with the same initial count of



(a)



(b)

Fig. 1 Bacterial viable count (**a**) and pH (**b**) during fermentation of apple juice by *L. plantarum* PCS 26, initiated at three different pH values

10^7 CFU/mL. The pH value of 3.5, which was indigenous for the commercial juice, did not support the growth of the culture, while the modified pH values did (Fig. 1a). The most intensive growth was attained at an initial pH of 5.1, leading to 1.3 x 10^{10} CFU/mL in only 11 h from the onset of fermentation. The culture growth was associated with an increase in the pH value rather than a decrease, as expected due to the lactic acid production (Fig. 1b). Preliminary testing of the sensory quality of this fermented apple juice demonstrated that the final product with a pH value about 7 was unacceptable in taste. Consumer sensory preferences are not adapted to beverages with neutral pH, since most of fruit juices are acidic (Kappes et al. 2007). The stability of the product during storage is expected to be negatively affected by this high pH value (Adams and Nout 2001). A relatively high cell number of 2.5 x 10^9 CFU/mL was achieved when the fermentation was initiated at pH 4.2. After 34 h, the apple juice reached its final pH value of 4.7. The sensory evaluation of this fermented apple juice made it evident that the product had a much better taste than the previous one, and was quite comparable to commercial fruit juices. Therefore, further fermentations were initiated at pH 4.2. Figure 2 shows the fit of the modified Gompertz model to the experimental data obtained from fermentation

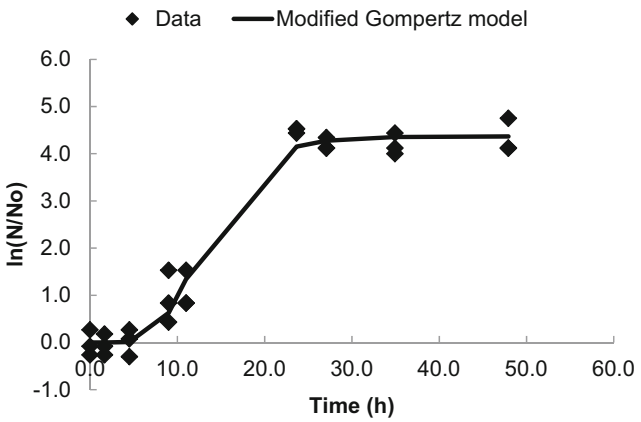


Fig. 2 Fit of the modified Gompertz equation to the growth of *L. plantarum* PCS 26 in apple juice initiated at pH 4.2

initiated at pH 4.2. This model was established as the best fit according to Akaike Criterion, and hence it was used to calculate the kinetic parameters. The estimated specific growth rate was 0.40±0.10 h^{-1}, the lag phase was 7.64±0.77 h, and the maximum growth was estimated as 1.9 log cycles.

Sugar consumption

The initial concentrations of the most abundant sugars in the apple juice, fructose, glucose and sucrose were 41.9±3.6, 29.3±3.2 and 6.7±0.7 g/L, respectively. The concentrations of these sugars did not change significantly during the fermentation (data not shown). This implicated that the culture was consuming them in small quantities below the standard deviation of the experimental data. Malolactic conversion and consumption of other substances that were not measured but were present in the juice could be responsible for growth, in the form of energy and carbon sources. Passos et al. (2003), in their research on growth kinetics of *L. plantarum* MOP-3, found out that the biomass yield coefficient (mg cells/mmol hexoses) was significantly increased while the maintenance coefficient (mmol hexoses/mg cells h) was significantly reduced in the presence of malic acid, indicating that the culture had lower demand for hexoses. Similar results were published by Plumed-Ferrer et al. (2008), who compared the growth of two strains of *L. plantarum*, both in MRS broth and in cucumber juice. They reported that the strains grown in cucumber juice showed relatively low hexose consumption of around 10 %, even though the media contained an excess of glucose. The reason for this behavior was recognized to be the utilization of malate present in the juice. The growth that they observed was from 10^8 to 10^{10} CFU/mL. When compared to the growth of our strain of *L. plantarum*, from 10^7 to 10^9 CFU/mL, even lower amounts of sugars are expected to be consumed. Using the HPLC method, the relative standard deviation of the experimental data for fructose, glucose and sucrose was around 10–11 %, which might well be the reason for not noticing the changes in their concentrations.

Malolactic fermentation

Figure 3 shows the change of malic acid and lactic acid concentrations in the apple juice during the fermentation. Malic acid and lactic acid were naturally present in the apple juice at concentrations of 7.9 and 1.8 g/L, respectively. In the course of fermentation, the culture was consuming malic acid until its complete depletion, and concurrently producing lactic acid, the concentration of which reached 7.7 g/L at the 37th hour of the fermentation (stationary phase). This process is described as malolactic conversion, by which the culture deacidifies the juice by converting the "harsher" diprotic malic acid into the softer monoprotic lactic acid (Reuss et al. 2010). The different structures of malic acid and lactic acid led to a reduction of titratable acidity and to an increase in the pH value. The conversion is mediated by a single malolactic enzyme, and releases carbon dioxide. Researchers have found that bacteria are able to derive energy from this process, even though it does not involve phosphorylation on the substrate level. A detailed study on the mechanisms of *L. plantarum* energy transduction by malolactic conversion was published by Olsen et al. (1991). In the literature, however, another metabolic pathway for the conversion of malate was revealed. Malate can be transformed into pyruvate by the so-called malic enzyme (malate dehydrogenase), and then converted to ethanol and acetate (Landete et al. 2010). This metabolism allows the culture to use malate as a carbon source as well. Thus far, only a few lactic acid bacteria have been found to convert the malate in this way: *Lactobacillus casei*, *Enterococcus faecalis* and *Streptococcus bovis* (Lahtinen et al. 2011; Landete et al. 2013). However, Garcerci Garcera et al. (1992) have also found that *L. plantarum* CEST 220 can grow on chemically defined media with malate as the sole energy source.

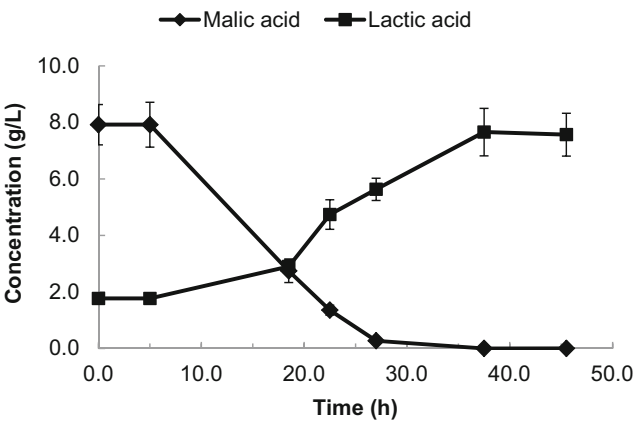


Fig. 3 Concentrations of malic acid and lactic acid during fermentation of apple juice by *L. plantarum* PCS 26 initiated at pH 4.2

Bacteria prone to malolactic conversion are much required in the wine industry (Bravo-Ferrada et al. 2013; Lasik 2013). Specially selected starter cultures, e.g., *L. plantarum* DSM 4361 and CNCM I-2924, that do not ferment major amounts of sugar (glucose and fructose) to lactic acid in the presence of malic acid are patented for the de-acidification of wine by malolactic conversion (Prahl 1994; Bou and Krieger 2012).

Effect of whey as a medium enhancer on bacterial cell growth

Figure 4 depicts the growth curves of *L. plantarum* PCS 26 in apple juice supplemented with 5, 10 and 15 %v/v whey. As can be noticed, the addition of whey markedly influences the growth of the culture. The modified Gompertz model was also fitted to these data and the estimated kinetic parameters are presented in Table 1. Apple juice supplemented with 5 %v/v whey resulted in a threefold higher specific growth rate and maximal growth that is twice as high compared to the pure apple juice. At the 19th hour, which can be distinct as the end of the exponential phase, the culture attained 5.0 x 10^{10} CFU/mL. Further supplementation with whey (10 and 15 %) did not result in better growth of the culture. Although the specific growth rate estimates were higher, the length of the lag phase increased substantially. Also, the onset of the stationary phase started earlier, (15th hour), and the maximal growth was lower. All this indicated that the culture has exhausted some essential nutrients present in the apple juice, which were diluted by adding more whey.

The increased growth with whey as compared to pure apple juice can be explained by the fact that lactic acid bacteria are fastidious microorganisms adapted to the nutrient-rich milk environment, where *L. plantarum* PCS 26 was isolated from. The downside of the whey addition is the introduction of lactose. According to the European Food Safety Federation (EFSA), the vast majority of subjects with lactose intolerance can tolerate up to 12 g of lactose as a single dose with no or minor symptoms (EFSA Panel on Dietetic Products 2010). The presence of 5 %v/v whey (around 3.5 % lactose) in the apple juice would result in up to 0.45 g lactose per 250 mL, a common serving size for beverages. Hence, the final product could still be considered suitable for lactose intolerant consumers.

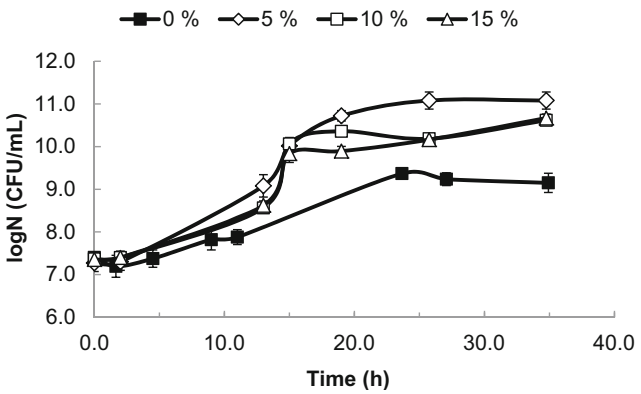


Fig. 4 Growth of *L. plantarum* PCS 26 during fermentation of apple juice supplemented with 5, 10 and 15 %v/v whey, initiated at pH 4.2

Table 1 Estimated kinetic parameters by modified Gompertz model for growth of *L. plantarum* PCS 26 on apple juice supplemented with whey*

Kinetic paremeter	0 % whey	5 % whey	10 % whey	15 % whey
Maximal growth (log cycles)	1.90±0.06	3.78±0.06	2.89±0.03	2.54±0.01
Specific growth rate (h^{-1})	0.40±0.10	1.20±0.14	3.06±0.38	3.67±0.17
Lag phase (h)	7.64±0.77	9.51±0.49	12.11±0.13	12.19±0.13

*Presented values are means±standard deviations (n=3)

Fermentation with immobilized cells of *L. plantarum* PCS 26

The growth of immobilized cells as well as their migration and growth in the medium (free cells) are presented in Fig. 5. The exponential phase of the growth curve of immobilized *L. plantarum* PCS 26 peaked at the 18.5th hour, when the number of immobilized viable cells was 3.2 x 10^6 CFU/mL. The lag phase was 7.04±1.06 h, and the maximal growth reached 0.82±0.03 log cycles, while the specific growth rate was 0.29+0.09 h^{-1}, estimated by the modified Gompertz model. The migration of the cells from the alginate beads into the medium started as soon as the lag phase ended, and intensified after 18.5 h, resulting in a decrease of the immobilization efficiency coefficient, η (Fig. 5). After 27 h, the concentration of free cells became almost level with the concentration of entrapped cells. The highest total viable count of 5.0 x 10^5 CFU/mL, corresponding to pH 4.7, was achieved at the 27th hour. The inferior growth compared to the free-cell fermentation was presumably due to the stress of the cells during

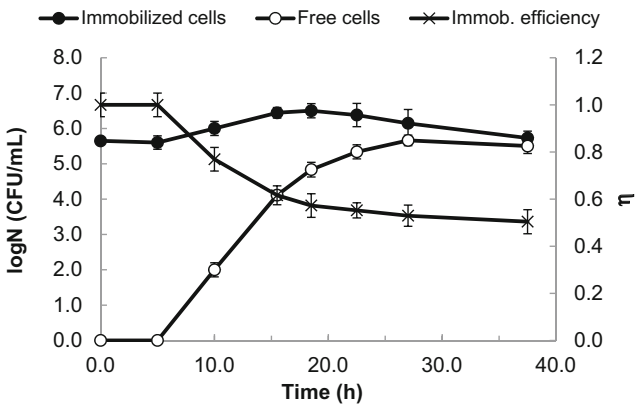


Fig. 5 Viable count of immobilized and migrated free cells of *L. plantarum* PCS 26 into the apple juice during fermentation with immobilized cells, initiated at pH 4.2. Immobilization efficiency, η, is a ratio between immobilized and total (immobilized plus free) number of cells

the entrapment and the slower mass transfer through the alginate. Taking into account that the average pore diameter of the alginate gels is around 20 nm (Klein et al. 1983) and the average diameter of *Lactobacillus* cells vary from 0.75 to 0.95 μm (Kokkinosa et al. 1998), degradation of the alginate would be the most probable reason for the intensive release of the cells into the medium. One of the possible mechanisms responsible for leakage of the cells could be degradation of the alginate due to the presence of phosphates. Data from the literature demonstrate that phosphates can induce fast degradation of the Ca-alginate gels (Bajpai and Sharma 2004). KH_2PO_4 solution can even be used for intentional depolymerization of the beads and release of the immobilized cells when measuring the viable count (Zerajic et al. 1990). The apple not only contains phosphates naturally (USDA 2003; Mita et al. 2013), but these compounds could also be added during production of apple juice to protect its color from enzymatic browning (Molins 1990).

Ca-alginate beads are commonly used as a support for cell immobilization because of their advantages, such as good biocompatibility, low cost, availability and easy preparation. Alginate itself is inert and non-toxic to the microorganisms, and thus is suitable for food applications. However, it is not the best choice for entrapment of the growing cells (Koyama and Seki 2004). Gel degradation, mass transfer limitations, low mechanical strength (causing cell migration through the gel) and large pore size are the main disadvantages associated with their use (Duarte et al. 2013). Further research on new immobilization materials and techniques should be undertaken to improve the growth kinetics and entrapment of *L. plantarum* PCS 26 in apple juice.

The stability of *L. plantarum* PCS 26 cells during storage

According to the Codex Alimentarius standard, a commercial probiotic beverage should possess a minimum viable count of 10^6 CFU/mL at the time of consumption (Codex Alimentarius Commision 2003). Probiotics must be consumed in amounts large enough to survive the upper digestive tract and to reach the intestine, where they can express their beneficial effect. Probiotic foods and beverages in which viable cells are better maintained will have a longer shelf life, which is very important for commercial products. The acidity of 4.7 (pH) puts the fermented apple juice at the borderline of being a highly acidic food (pH 4.6) (Sun-Young 2004). However, since it is not supposed to be pasteurized after the fermentation (live probiotic), the juice could be subjected to microbial infections, mostly fungal, if not properly handled. Therefore, this product should be stored at refrigeration temperatures, not only to extend the viability of the probiotic culture, but also to suppress the growth of other microorganisms.

Figure 6 presents the viable count of free and immobilized cells in the fermented apple juice during 17 days of refrigeration (4–7 °C). The Weibullian model for their survival was fitted to the data and the parameters "n" and "b" were determined. This model is empirical and there are no links between the model parameters and theories about the death of the microorganisms (van Boekel 2009). The parameter "n" for the free cells was higher than one (n=2.1±0.13) which means, as is obvious from Fig. 6a, the remaining cells become increasingly susceptible to the harsh environment and the survival rate decreases during the storage time. On the other hand, the immobilized cells (n=0.27±0.02) rapidly decreased in viability in the beginning, but the remaining cells adapted to the stress (Fig. 6b). Storage times by which the probiotic juice would retain a viable count of above 10^6 CFU/mL were predicted for free and immobilized cells, by using the estimated model parameters and the initial cell count before storage. The storage time for the free cells with an initial cell concentration of 2.5 x 10^9 CFU/mL was 22.0±0.68 days (inactivation of log 3,.4) while for the immobilized cells, whose initial cell concentration was 3.2 x 10^6 CFU/mL, was 32.1±5.2 days (inactivation of log 0.6).

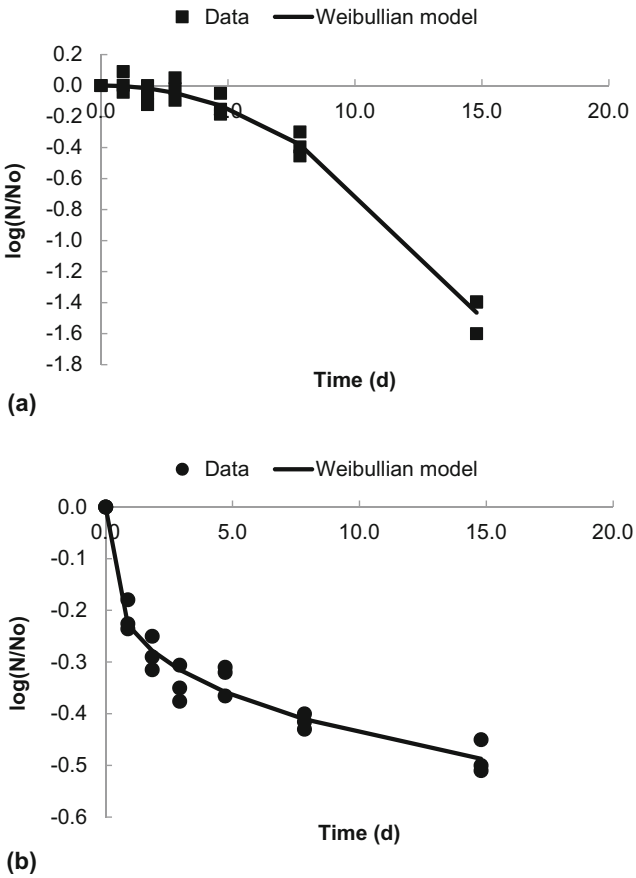


Fig. 6 Weibullian model fit to the viable count data of free cells (**a**) and immobilized cells (**b**) in apple juice during refrigeration storage at 4–7 °C. **a** b=0.005±0.002, n=2.2±0.13; **b** b=0.24±0.01, n=0.27±0.02

The survival of the probiotic cultures after fermentation differs among studies, and ranges from a day to a maximum of 4 weeks (Prado et al. 2008). Research has been done on

Table 2 Sensory evaluation of apple juice fermented with *L. plantarum* PCS 26

Fermentation	Initial pH	Smell	Color	Transparency	Taste
Free cells	pH 5.1	very good	acceptable	acceptable	unacceptable
Free cells	pH 4.2	very good	acceptable	acceptable	acceptable
Free cells, supplementation with 5 %v/v whey	pH 4.2	very good	acceptable	acceptable	acceptable
Immobilized cells	pH 4.2	very good	very good	very good	acceptable

optimizing the composition of the fermenting material to assure high probiotic survival throughout the shelf life of the product (Bernat et al. 2014b). Working on apple juice, Ding and Shah (2008) got similar results for the survival of free and microencapsulated *L. plantarum* bacteria. However, in their study, apple juice served only as a carrier for the culture, rather than as a medium for fermentation. Since *L. plantarum* PCS 26 exerts probiotic effects through its extracellular components as well (Dimitrovski et al. 2014), fermentation of the apple juice with this bacteria was essential. Only then could the strain secrete its metabolites into the medium—apple juice. It should be also noted that fermentation with probiotic bacteria could simplify the production technology and reduce costs in comparison to the general practice of inserting probiotics into pre-fermented products.

Preliminary sensory evaluation

The preliminary sensory evaluation was performed to eliminate the sensory-unacceptable fermented apple juices, rather than to thoroughly assess all fermented products. The results are summarised in Table 2. According to the panelists, the smell did not significantly change between the fermentations. The color of fermented apple juice was slightly darkened, but still attractive to the eye. The transparency of the free-cell fermentations was decreased, but it was graded as acceptable. The apple juice with 5 %v/v whey had a slight milky taste and its transparency was the lowest of all. The Ca-alginate beads were visible in the immobilized-cell juice and caused a minor swallowing discomfort. The apple juice fermented at an initial pH value of 4.2 had the most pleasant taste in comparison to commercial juice.

Conclusions

The lactic acid bacterium *L. plantarum* PCS 26 was successfully used to create fermented, non-milk based probiotic beverages. The apple juice with 5 %v/v whey yielded the best fermentation results in terms of the highest specific growth rate and the maximal cell concentration. Immobilized cells had deprived growth because of the entrapment stress, but the stability during shelf life at temperatures of 4 to 7 °C exceeded by far the survival of the free cells. As estimated by the Weibull model, the concentration of the live bacteria in the juice would retain their critical value of 10^6 CFU/mL for about 30 days. Future studies should focus on other immobilization techniques, as well as detailed sensory assessment of the fermented apple juices.

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