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ORIGINAL ARTICLE

Transfer and subsequent growth and metabolism of *Lactobacillus plantarum* in orange juice medium during storage at 4 and 30°C

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Lactobacillus plantarum, metabolism, oranges, temperature, transfer.

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Abstract

Aim: To investigate the physicochemical changes produced from growth and metabolism of *Lactobacillus plantarum* N4 in orange juice medium stored at 4 and 30°C after transferring from artificially inoculated oranges peal during extraction.

Methods and Results: Lower than 2·0% of total of the N4 strain was recovered in juice extracted from inoculated oranges (about of 10^9 CFU ml^{-1}) under assayed conditions. After that, the N4 strain grew 2·43 ± 0·09 log cycles in 48 h at 30°C. Sugars such as glucose and fructose and L-malic and citric acids were utilized, although at different rates and extent, yielding significant lactate and acetate amounts with a concomitant pH reduction. Ethanol, diacetyl, acetoin or 2,3 butilenglicol were undetected. During juice storage at 4°C bacterial counts, sugars composition and pH remained significantly unchanged as well as its sensory attributes.

Conclusion: The transfer rate of *L. plantarum* N4 to freshly squeezed juice under adequate hygienic condition was low. At 30°C, the micro-organism rapidly initiated growth, producing acids but not butter flavour compounds neither ethanol.

Significance and Impact of the Study: The ability of this strain to survive in refrigerated juice without cause spoilage warrants further investigation to explore its potential use for biotechnology applications.

Introduction

Fresh orange juice is successful on the market because of its taste and nutritional value. It is obtained from mature citrus fruit that has not been further pasteurized, frozen or concentrated after extraction (Zanoni *et al.* 2005). Typically, the surface of fresh picked citrus contains about 3·6 ± 0·3 log CFU cm^{-2} of total aerobic organisms. Processing, although minimal, may increase microbial spoilage in the fruit juice by transferring skin microbiota to fruit flesh where micro-organisms could grow rapidly (Pao and Davis 2001). In general, the low pH of fruits juice restricts microbiota to acid-tolerant micro-organisms such as fungi and lactic acid bacteria (Andrés *et al.* 2004). Arena *et al.* (1999) reported that *L. plantarum* N4 and N8 isolated from oranges peal were able to derive energy and ammonia from arginine or citrulline catabolism, which is interesting for micro-organisms developing in a stressful environment. Saguir *et al.* (2008) demonstrated the ability of *L. plantarum* N4 to assimilate mainly dipeptides rather than amino acids in nutritionally deficient condition. However, little information is available about its behaviour and metabolic activity in fresh juice when stored at different temperatures. Tajchakavit *et al.* (1998) reported that *Lactobacillus* and *Leuconostoc* spp. can multiply in apple juice causing production of off-flavour compounds (diacetyl), gas, slime and changes in acidity. Traditional thermal treatments application can ensure the microbiological stability of refrigerated fruit juice, but concomitant quality losses in terms of nutritional and sensorial attributes also occur (Perez-Cacho and Rouseff 2008). In unpasteurized orange juice, the

final product characteristics are especially dependent on storage temperature (Zanoni *et al.* 2005). Although it is usually refrigerated, sometimes it is displayed at room temperature enhancing its bacterial amplification risk. Gardner *et al.* (2001) evaluated lactic acid bacteria for use in vegetables fermentation to enhance the microbial stability and quality of final product. On the other hand, fruit juice can serve as health-promoting micro-organisms carrier, if precautions are taken in regards with sensory characteristics and pH. However, research of this matric as raw material for probiotic bacteria is still scarce (Nualkaekul and Charalampopoulos 2011). Because *L. plantarum* is a potential contaminant of orange juice, a more accurate knowledge on its capacity to infect fresh juice and subsequent behaviour can improve our understanding on its detrimental effect to quality and alternatively can provide information in direction to a potential biotechnology application. Therefore, this study was performed to investigate the ability to transfer of *L. plantarum* N4 from inoculated oranges peal to fresh juice during extraction process and, subsequent influence of growth and sugars and organic acids metabolism by the transferred population on chemical composition of the natural medium during storage at 4 and 30°C.

Materials and Methods

Preparation of culture

Lactobacillus plantarum N4 isolated from orange peel (Arena *et al.* 1996) was used in this study. This micro-organism was stored at −20°C in MRS broth (De Man *et al.* 1960) supplemented with glycerol (30%, v/v). *L. plantarum* N4 was propagated in MRS broth and incubated without agitation at 30°C for 8 h. After that, cells were harvested by centrifugation at 7000 ***g*** for 15 min, washed twice and resuspended in sterile distilled water to give a cell density of 9·28 ± 0·38 log CFU ml^{-1}. A separate cell suspension was grown for each replicate of each experiment.

Preparation of fruit

Sweet oranges (*Citrus sinensis*) obtained from local packinghouses before washing were used in this study. The products were all of agreeable sensory quality. Fruits were disinfected with an ethylic alcohol solution (70% v/v), then rinsed twice with sterile distilled water to eliminate the background microbiota and allowed to reach room temperature. Fruits were then completely immersed in the inoculum for 15 or 30 min. Inoculated oranges were drained for 30 min after which the juice was extracted. Before extraction, an inoculated oranges group was separated for *L. plantarum* enumeration and used another group ($n = 5$) for the transfer experiment, assuming they carried the same number of bacteria. Noninoculated washed oranges were used as control.

Orange microbial load

Oranges and skin of noninoculated and inoculated ones after cleaning treatment were washed three times with sterile distilled water. Each water washing was collected under sterile conditions and immediately used for microbial evaluation. 0·1 ml aliquots of serial decimal dilutions were plated in duplicate on MRS agar medium acidified to pH 5·0 and supplemented with 1·3 $\mu g\ ml^{-1}$ of Pimaricin (Sigma) (MRS-P) to inhibit yeasts growth and in the same medium enrichment with fructose (20 $g\ l^{-1}$) and citric acid (4 $g\ l^{-1}$), (MMRS-P). Total aerobic mesophilic flora was determined on plates count agar (PCA, Oxoid, Basingstoke, UK). Agar plates were incubated anaerobically, MRS-P and MMRS-P plates (BBL GasPak Anaerobic System), and aerobically, PCA plates, at 30°C for 7 day before enumeration. The PCA and MRS-P agar plates were flooded after enumeration with 3% H_2O_2 to observe for the presence of catalase-positive colonies. For differentiation of *L. plantarum*, 60 colonies on MRS agar plates were picked randomly and identified by means of partial amplification product comparison of the recA gene by PCR multiplex (Torriani *et al.* 2001), which was performed as described by Savino *et al.* (2011).

Bacterial transfer during juice extraction

Inoculated oranges were cut in halves and squeezed to obtain the juice. A small-scale commercial juice extractor (Citrus Press 1000, Type HR2783/A, Philips) was used for the experiment under sterile conditions. All instruments and equipment were washed and sanitized with 0·25 $g\ l^{-1}$ chlorinated water (Ayhan *et al.* 1998). Juice samples were immediately used (within 15 min) for microbial evaluation after extraction and then stored at 4 and 30°C for 4 weeks. Juice samples obtained from washed noninoculated fruits were evaluated as control.

The rate of transfer (TR) was calculated as described by Chen *et al.* (2001) using:

$$\mathrm{TR}(\%) = \frac{\mathrm{CFUrecipient}_{(\mathrm{juice})}}{\mathrm{CFUdonar}_{(\mathrm{orange\ surface})}} \times 100$$

L. plantarum enumeration in juice

Growth was evaluated by the estimation of the number of viable bacterial cells at various times. The cultures were serially diluted in 0·1% peptone water; 0·1 ml of the suspension was then spread onto MRS agar medium in

duplicate. The plates were aerobically incubated at 30°C for 3 days, after which they were counted and expressed as log CFU ml^{-1}. pH was analysed using a pH meter (HANNA instruments, Milan, Italy).

Determination of organic acids, sugars and ethanol

HPLC analyses were carried out for the glucose, fructose, organic acids and ethanol determination using an ISCO liquid chromatograph (ISCO, Lincoln, NE) as described by Sajur *et al.* (2007). D-glucose was also measured by the glucose oxidase method (Wiener Laboratory, Rosario, Argentina). Diacetyl, acetoin and 2,3-butanediol was analysed as a combined value according to the colorimetric method of Branen and Keenan (1970).

Statistical analysis

Results were expressed as means ± standard deviations of at least two independent experiments. To validate the methods, Student's *t*-test was used.

Results

Transfer of *L. plantarum*

Oranges surface prior to washing treatment contained levels of 3·5 ± 0·12 log CFU ml^{-1} determined on PCA plates being majority of colonies positive catalase belonging to yeasts. Lower numbers (in order 10^2 CFU ml^{-1}) were found of small, negative catalase colonies on MRS-P or MMRS-P plates, suggesting they were lactic acid bacteria. After washing procedure, microbial growth was not detected.

In inoculated oranges surface, the microbial counts ranged from log 9·12 ± 0·22 to 9·21 ± 0·23 log CFU ml^{-1} for a 15- and 30-min inoculation time, respectively, being constituted by the inoculated bacterium (Gram-positive and catalase-negative rods). In addition, the PCR multiplex assay confirmed these results because all tested colonies gave a single product of 318 bp, the expected size of the PCR fragment for *L. plantarum*. A small portion of the N4 strain on fruit was introduced to fresh juice during extraction under assayed conditions. Thus, bacterial levels detected in the juice samples were of about 2 log lower than level found on fruits. This represented an average TR of 1·05 ± 0·23% (Table 1). No microbial transfer from uninoculated samples to fresh juice was detected by plating procedure.

Cell survival and growth in orange juice

The N4 strain growth in fresh juice and pH variations at 4 and 30°C for 7 days are shown in Fig. 1. After this time, similar results were obtained until 4 weeks of incubation (Data not shown). Juice samples obtained from noninoculated washed oranges stored under the same conditions were used as control. The average initial count of *L. plantarum* N4 corresponding to 6·90 ± 0·12 log CFU ml^{-1} and the initial pH of juice remained constant without decreasing at any time during storage at 4°C. By contrast, the N4 strain grew with a growth rate of 0·096 h^{-1} and reached a maximum cell density of 9·22 ± 0·19 log CFU ml^{-1} at 3 days at 30°C. After this

Table 1 Counts of *Lactobacillus plantarum* N4 on inoculated oranges at two inoculation times prior to extraction and of the transferred population to fresh juice during extraction process

Time inoculation (min)*	Inoculated oranges	Juice from inoculated oranges	
	Log CFU ml^{-1}	Log CFU ml^{-1}	% transfer†
15	9·12 ± 0·11	6·90 ± 0·12	0·54 ± 0·11A
30	9·23 ± 0·25	7·33 ± 0·29	1·56 ± 0·44A

*During this time, the oranges were immersed in the cell suspension of *L. plantarum* N4 (in order of 10^9 log CFU ml^{-1}) before drained and juice extraction process.

†Percentage of *L. plantarum* N4 transferred from the inoculated oranges surface to juice. Values with the same letter in the same column were not significantly different ($P < 0·05$).

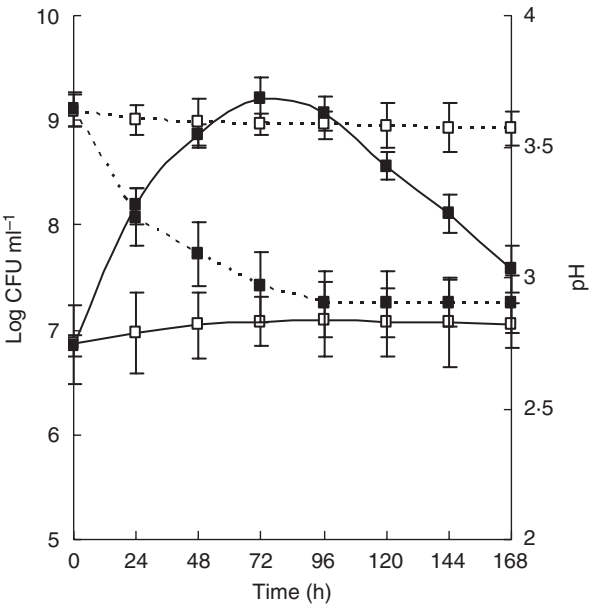

Figure 1 Evolution of *Lactobacillus plantarum* N4 (—) and variation of pH (.....) in orange juice medium obtained from inoculated fruits during 7 days of storage at 4°C (□) and 30°C (■). In control samples, no growth and significant changes in pH occurred at storage both temperatures.

time, it began to decrease progressively to reach a final cell density of 7·88 ± 0·22 log CFU ml^{-1} at 7 days, and then cell counts remained unchanged. Initial pH decreased from 3·60 ± 0·06 to 2·90 ± 0·11 for 4 days at 30°C (Fig. 1). In this condition, some alteration sign (visual, odour) was observed at 5 days.

In all cases, no bacterial growth and significant changes in pH ($P \leq 0{\cdot}05$) occurred in control media. When initial population corresponded to 7·33 ± 0·29 log CFU ml^{-1}, similar results were observed.

Bacterial metabolism in orange juice

Table 2 presents the chemical changes that took place in the fruit juices containing *L. plantarum* N4 after 4 weeks of storage at 4°C, including the pH, main sugars and organic acids evolution. Only residual concentrations of L-malic and citric acids decreased at 4 weeks of incubation and represented 63·1 and 54·3% of their initial levels, respectively. The organic acids utilization was accompanied by an increase in concentration of lactic and acetic acids in a molar ratio of 0·96–0·68 mmol $mmol^{-1}$, respectively, although acetic acid was produced accounting for 89% citric acid degraded.

At 30°C of storage, the utilization of sugars (glucose and fructose) and L-malic and citric acids began immediately after the growth began, the major part of consumption being in the first 2 days. Sugars were consumed slower thereafter and finally stopped before complete depletion at 3 days. No sucrose was degraded. Maximum consumptions corresponded to 31·4, 19·3 and almost 100% of initial glucose, fructose and organic acids, respectively, coinciding with the end of bacterial growth. At this time, significant amounts of lactic and acetic acids were produced by the transferred micro-organism (Table 3). The analytical balance of lactate from glucose + fructose consumed was 3·5 mmol $mmol^{-1}$, almost twice higher than the theoretical expected value for homolactic behaviour. The additional 74·1 mmol l^{-1} could come from the organic acids catabolism with a percentage of carbon recuperation up to 100%. Acetic acid was produced, accounting for 40% substrates degraded. However, it was formed accounting for 96% citric acid consumed, sugaring that acetic acid was derived exclusively from citrate. None of ethanol and aroma compounds (diacetyl, acetoin and 2,3-butanediol) were formed during storage. The pH diminution in juice was correlated with the organic acids production,

Table 2 Changes in sugars and organic acids concentrations in orange juice medium obtained from inoculated oranges with *Lactobacillus plantarum* N4 during storage at 4°C

	Sugars and organic acids (mmol l^{-1})†						
Sample	Glucose	Fructose	Sucrose	Malic acid	Citric acid	Lactic acid	Acetic acid
Week 0	98·0 ± 4·4	104·1 ± 2·5	129·2 ± 3·1	11·1 ± 0·6	44·2 ± 0·6	0	0
Week 4	93·1 ± 3·7	107·8 ± 3·4	124·1 ± 3·4	4·1 ± 0·1*	20·2 ± 0·1*	29·8 ± 0·4*	21·4 ± 0·5*

†Each number is the mean of two independent experiments taken from different experiments (coefficient of variation <5%). Values with asterisk (*) in the same column were significantly different ($P \leq 0{\cdot}05$).

Table 3 Changes in sugars and organic acids concentrations in orange juice medium obtained from inoculated oranges with *Lactobacillus plantarum* N4 during storage at 30°C.

	Sugars and organic acids (mmol l^{-1})†						
Sample‡	Glucose	Fructose	Sucrose	Malic acid	Citric acid	Lactic acid	Acetic acid
Day 0	98·0 ± 4·2	105·0 ± 3·5	129·2 ± 3·2	11·30 ± 0·6	44·2 ± 0·6	0	0
Day 1	87·1 ± 3·8	96·0 ± 3·2	128·8 ± 4·6	4·10 ± 0·20	24·5 ± 0·2	88·9 ± 2·1	21·0 ± 1·2
Day 2	74·8 ± 3·7	89·7 ± 3·1	127·1 ± 3·8	0·97 ± 0·03	12·2 ± 0·5	144·4 ± 3·1	31·3 ± 1·4
Day 3	70·3 ± 3·1*	86·6 ± 2·0*	127·0 ± 4·7	0·21 ± 0·01*	2·3 ± 0·1*	166·0 ± 3·6*	37·9 ± 2·1*
Day 4	68·0 ± 2·7	86·6 ± 2·6	126·1 ± 4·1	0·11 ± 0·01	1·9 ± 0·1	173·0 ± 3·8	40·8 ± 1·7
Day 5	67·0 ± 2·1	86·6 ± 1·8	126·1 ± 4·4	0·10 ± 0·01	1·9 ± 0·1	173·0 ± 3·6	40·5 ± 1·6
Day 6	67·0 ± 2·5	86·6 ± 2·6	126·1 ± 4·6	0·10 ± 0·01	1·9 ± 0·1	173·2 ± 3·9	40·8 ± 1·8
Day 7	67·0 ± 2·6	86·5 ± 2·1	126·1 ± 3·8	0·10 ± 0·01	1·9 ± 0·1	173·0 ± 3·7	40·8 ± 1·6

†Each number is the mean of two independent experiments taken taken from different experiments (coefficient of variation <5%). Values with asterisk (*) in the same column were significantly different as compared to those detected at day 0 ($P \leq 0{\cdot}05$).
‡After 7 days of storage, similar results were found.

which was significantly higher than that observed in refrigerated juice.

Discussion

The results obtained confirmed the effectiveness of the employed cleaning procedure to reduce the microbial load of orange peel, mainly constituted by yeasts. No micro-organism was transferred from uninoculated washed oranges surface to fresh juice during extraction, confirming the importance of the adoption of good hygiene and handling practices during fruit processing (Nunes *et al.* 2010). The TR of *L. plantarum* N4 from inoculated oranges surface with 10^9 CFU ml^{-1} to fresh juice was low in concordance with the results described by Pao and Davis (2001). Many factors may contribute to bacterial transfer from surfaces to food such as food composition and surface type (Dawson *et al.* 2007). Thus, orange surface could play an important role in decreasing bacterial juice contamination during processing.

Lactobacillus plantarum N4 transferred to squeeze juice survived during all storage period at 4°C. L-malic and citric acids were partially metabolized, indicating that they could be used as energy source for cell maintenance as reported by Vaningelgem *et al.* (2006), without producing significant changes in pH and sensorial attributes of juice. The fact that refrigerated fresh juice supported well the *L. plantarum* N4 survival without causing spoilage was an interesting finding in view of its potential biotechnological application for novel functional juice manufacture. Chon and Choi (2010) demonstrated the probiotic properties of *L. plantarum* isolated from fermented vegetable. Champagne and Gardner (2008) evaluated the survival of probiotic lactobacilli strains in a commercial fruit drink stored at 4°C. Luckow and Delahunty (2004) examined the sensory impact of functional ingredients, including probiotics on the aroma and taste of orange juices with acceptable results.

In the fresh juice stored at 30°C, the N4 strain rapidly grew; then, the viable cells number began to decrease which could be due to low pH and high acidity in the fermented juice. With regard to carbohydrate substrates utilization, the N4 strain preferred glucose rather than fructose for growth in concordance with Sajur *et al.* (2007). The high residual sugar levels obtained at the end of bacterial growth suggested that the fermentation stopped because of low pH rather than because of carbohydrate substrates lack. On the other hand, the result of fermentation balance of lactic acid formed from substrates degraded supports the idea that lager sugars amounts could have been utilized by the N4 strain as compared to the experimental results. Nualkaekul and Charalampopoulos (2011) demonstrated that sugars increased significantly in orange juice containing *L. plantarum* during storage at 4°C, presumably due to the enzymatic hydrolysis of polysaccharides. Citric acid was mainly utilized by the N4 strain during growth in fresh juice. Sánchez *et al.* (2008) showed the beneficial effect of citrate on *Lactococcus lactis* growth under acid stress condition, which stemmed from less expenditure of ATP, derived from glucose catabolism, to achieve pH homoeostasis. Saguir and Manca de Nadra (2002) demonstrated in *Oenococcus oeni* from wine the beneficial effect of citrate and L-malate for the growth rate, the biomass formed and to fill in the amino acid requirements. It was noticeable that no diacetyl, acetoin and 2,3 butanediol neither ethanol were produced by the N4 strain in fermented orange juice at 30°C. Thus, the changes in organoleptic properties of juice at abusive temperature were related to the organic acids quantities produced at 4/30°C. Diacetyl is considered an off-flavour compound in fruits, vegetables and derivates. Gardner *et al.* (2001) determined that the *Lactobacillus* strains that formed less diacetyl produced the most favourable fermented carrot juice. Thus, *L. plantarum* N4 homofermentative could be used for application in acid vegetables fermentation, which is expected to become each more important in preserving them for feeding humanity.

In conclusion, the probability of *L. plantarum* to contaminate freshly squeezed orange juice under adequate hygienic practice was low. At 30°C, *L. plantarum* grew spoiling the fresh juice by producing high lactic and acetic acids concentrations but not butter flavour compounds neither ethanol. In the refrigerated juice, the ability of the N4 strain to survive without causing spoilage during storage warrants further investigation to explore its potential use in a biotechnology application.

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