Apple, grape or orange juice: Which one offers the best substrate for lactobacilli growth? - A screening study on bacteria viability, superoxide dismutase activity, folates production and hedonic characteristics

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Apple, grape or orange juice: Which one offers the best substrate for lactobacilli growth? — A screening study on bacteria viability, superoxide dismutase activity, folates production and hedonic characteristics

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A B S T R A C T

Fermentation can contribute to improve functional aspects of foods. The first goal of this study was to determine amongst apple, grape and orange juices, the one with the best bacterial growth performance during fermentation by *Lactobacillus* strains from commercial and artisanal food origins, at 40 °C for 48 h. The juice with the highest bacterial growth was evaluated for bacteria viability during 4 weeks of cold storage, superoxide dismutase (SOD) activity and folates production analyzed through HPLC/fluorimetry. Acceptability of fermented juice was appraised through hedonic analysis. Lactobacilli counts were the highest in apple and the lowest in orange juices at t = 48 h. In most cases, bacteria counts were higher in fermented (5.5 to 9.5 log CFU/ml) than in supplemented apple juices (4.2 to 5.7 log CFU/ml), at the 4th week of cold storage. SOD activity was significantly increased in all apple juices fermented by commercial Lactobacilli strains. Folates were produced in apple juices fermented by *Lactobacillus plantarum* and *Lactobacillus rhamnosus*. Apple juice was the best substrate for *Lactobacillus* growth and, considering bacterial viability and overall acceptance by the panelists, *Lactobacillus acidophilus* L10 was the most suitable strain for apple juice fermentation.

Keywords:
Lactobacillus
Fruit juice fermentation
Folate
Antioxidant enzyme
Acceptability test

Chemical compounds studied in this article:
Acetonitrile (PubChem CID: 6342)
p-Aminobenzoic acid (PubChem CID: 978)
Formic acid (PubChem CID: 284)
2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2 *H*-tetrazolium, monosodium salt (PubChem CID: 11,399,451)
5-Methyltetrahydrofolic acid diglutamate (PubChem CID: 40,114)
5-Methyltetrahydrofolic acid monoglutamate (PubChem CID: 42,626,431)
Sodium chloride (PubChem CID: 5234)

1. Introduction

An important portion of the market of functional foods is represented by foods containing probiotics (Siegrist, Stampfli, & Kastenholz, 2008), which have been defined as live microorganisms that, when consumed in adequate amounts, confer health benefit on the host (FAO/WHO, 2006). This concept has been systematically debated by the European Food Safety Authority (EFSA), which remarks the lack of irrefutable scientific proof of 'beneficial physiological effect' of many probiotic claims (Katan, 2012). However, the market of probiotic food continues to grow at rates of 7%/year worldwide (Foligné, Daniel, & Pot, 2013). Dairy matrices, markedly yoghurt, have been systematically studied and commercialized as vehicles for probiotics intake (Granato, Branco, & Nazzaro, 2010). However, in addition to the needs of vegan diet consumers, the high prevalence of lactose intolerance in worldwide population is boosting the diversification of the delivery vehicles available, beyond the traditional use of dairy systems (Espirito Santo, Perego, Converti, & Oliveira, 2011). In this sense, fruit juices are perceived as healthy food product by consumers and have been suggested as an appropriate medium for supplementation with probiotic bacteria, being the species of Lactobacilli the most used for *probiotication* (Espirito Santo et al., 2011; Martins et al., 2013). Nevertheless, the development of non-dairy products containing probiotic is a challenge, as their viability is highly dependent on factors inherent to the food matrix and to food process such as base nutrients, pH, presence of inhibitor substances, oxygen level and

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temperature of storage (Champagne, Raymond, & Gagnon, 2008; Mattila-Sandholm, Myllärinen, Crittenden, Mogensen, & Fondén, 2002). In this context, the choice of the probiotic bacteria strain, the best adapted to a given substrate, is of outmost importance (Ranadheera, Baines, & Adams, 2010).

Apart from supplementation, the fermentation of a food matrix by probiotic cultures consists in another way of *probiotication* which can contribute to further technological and/or health benefits (Rakin, Vukasinovic, Siler-Marinkovic, & Maksimovic, 2007; Stanton, Ross, Fitzgerald, & Van Sinderen, 2005). Beyond the probiotic claim itself, metabolites produced during fermentation by probiotic bacteria can be responsible for the improvement of functional aspects of the foods (biogenic effect), amongst which one can mention the production of B-group vitamins such as folates (Jägerstad, Jastrebova, & Svensson, 2004; LeBlanc et al., 2013), functional fatty acids (Espírito-Santo et al., 2012) and anti-hypertensive peptides (Agyei & Danquah, 2012; Ankolekar, Pinto, Greene, & Shetty, 2012). As a response to oxidative stress during fermentation, species of lactobacilli and bifidobacteria can produce anti-oxidant substances such as NADH, NADPH, glutathione and superoxide dismutase (SOD) enzyme. These substances are able to decrease the risk of accumulation of reactive oxygen species (ROS), thus increasing the bacteria cell viability and the antioxidant capacity of the food (Lin & Yen, 1999; Wang, Yu, & Chou, 2006). The reduction of undesirable substances such as nondigestible oligosaccharides (Hou, Yu, & Chou, 2000) is also an advantage of fermentation.

Herein, apple, grape and orange juices were considered as substrate for fermentation by monocultures of *Lactobacillus* species, which are present in commercial and artisanal food products and have large scientific documentation of safety and health benefits (FAO/WHO, 2006; Galdeano & Perdigon, 2004; Gobbetti, Di Cagno, & De Angelis, 2010; Isolauri, Salminen, & Ouwehand, 2004; Jones, Tomaro-Duchesneau, & Prakash, 2014; Peran et al., 2007). The first goal was to determine and select the juice with the best bacterial growth performance during fermentation. Afterwards, considering cell viability during 28 days of cold storage, fermentation process was confronted to the simple supplementation of the chosen fruit juice with free lactobacilli strains. The influence of fermentation on pH, SOD activity, production of folates and hedonic characteristics of the selected fruit juice, were also appraised. This screening study is concentrated on the effect of each one of the bacteria strains tested on the food matrix (and vice-versa) during fermentation and is part of a larger project on the impact of fermentation on functional compounds of vegetable food matrices.

2. Materials and methods

2.1. Fruit juices

Microfiltrated apple juice of 'Golden Delicious' variety, was produced as described in Hubert, Baron, Le Queré, and Renard (2007) and purchased from IFPC (Institut Français des Productions Cidricoles, Le Rheu, France). Orange juice (Joker®) without pulp and grape *var.* 'Muscat' juice (Casino Bio®), both 100% fruit juice without pulp and additives or preservatives, were purchased in local supermarkets in Avignon, France. Dry matter contents of apple, grape and orange juices were 12.1 (±0.1), 16.6 (±0.2) and 11.5 (±0.2) g/100 ml, respectively.

2.2. Bacteria cultures

In this study we used freeze-dried commercial cultures of *Lactobacillus acidophilus* Lafti L10, *Lactobacillus casei* Lafti L26 (DSM, Moorebank, Australia) and *Lactobacillus rhamnosus* LGG (ATCC 53103), and cultures isolated from commercially available products: *Lactobacillus paracasei* Lp33 (Christian Hansen, Denmark), *Lactobacillus plantarum* 299v (Probi AB, Sweden). Lactobacilli strains isolated from artisanal food products and maintained in the INRA collection were also used: *L. acidophilus* (CIRMBIA 1674, CNRZ 204), *L. casei* (CIRMBIA 667, CNRZ 313), *L. paracasei* (CIRMBIA 672, CNRZ 62), *L. plantarum* (CIRMBIA 466, CNRZ 211) and *L. rhamnosus* (CIRMBIA 607, CNRZ 212). The identity of *Lactobacillus* species isolated from commercial products was confirmed through amplification of DNA from the 16S rRNA which were then compared to sequences deposited on BQ GenBank-EMBL by using BLASTN program of National Centre for Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov), and identified to a species with % of homology >97%.

2.3. Fermentation procedures and supplementation with lactobacilli strains

Lactobacilli cultures were previously inoculated into MRS (de Man, Rogosa, and Sharpe) broth (Biokar diagnostics, Beauvais, France) and incubated at 40 °C for 12 h. Then, the resulting cultures were adapted to the growth conditions of the fruit juices. The final cultures were centrifuged at 5000 ×*g* for 5 min and the resulting bacterial pellets were washed three times with a sterile solution of NaCl 0.9% (w/v) and once with the fruit juice. The resulting pellet was re-suspended in a volume of fruit juice enough to achieve bacteria counts of 10^9 CFU/ml, verified through culture on MRS agar (Biokar diagnostics, Beauvais, France) in petri dishes and incubation at 37 °C for 48 h under anaerobiosis.

Juices were divided into 10 ml aliquots in sterile BD Falcon® tubes of 15 ml, inoculated with 1% of inoculum at 10^9 CFU/ml and then incubated in water bath at 40 °C for 48 h, under agitation of 100 rpm. Tubes in triplicate were prepared for 8, 24 and 48 h of fermentation and for 1, 14 and 28 days of cold storage at 5 °C, after 48 h of fermentation. Tubes were retrieved at each established fermentation time and cooled until 20 °C in ice bath. The contamination by yeasts was controlled through immersion phase-contrast optical microscopy at ×1000 magnification (Olympus BX50, Olympus Optical Co., Ltd., Hamburg, Germany) periodically during fermentation. Independent batch fermentations were repeated twice. Juices without inoculum were maintained under the same fermentation conditions and used as control for all the analysis.

The evolution of pH during fermentation and during cold storage was controlled for each tube with a pH meter (Mettler Toledo FE20, Schwerzenbach, Switzerland) and pH electrode (Mettler Toledo Electrode LE438) calibrated with freshly prepared buffers (pH 4.00 and pH 7.01) (Merck, Darmstadt, Germany).

In order to evaluate whether or not the fermentation process contributes to better cell viability during shelf-life, the fruit juice presenting the highest bacteria counts by 48 h of fermentation was chosen to undergo a simple supplementation with the lactobacilli strains. The non-fermented selected fruit juice at 5 °C was inoculated with fresh cell suspension of lactobacilli strains at 9 log CFU/ml and kept for 4 weeks under cold storage at 5 (±1) °C after inoculation. Three independent replications of supplementation were performed.

2.4. Lactobacilli enumeration

Viable *Lactobacillus* counts were carried out in triplicate at 8, 24 and 48 h of fermentation and on days 1 and 28 of cold storage of fermented and supplemented juices. Appropriate serial decimal dilutions of samples (100 µl) of juices in sterile 0.9% NaCl (900 µl) were inoculated into MRS agar at pH 6.2, applying the pour plate technique, followed by incubation at 37 °C for 48 h under anaerobiosis. Afterwards, isolated colonies were counted and the results were expressed in log CFU/ml of juice.

2.5. Superoxide dismutase activity assay

The determination of SOD activity was carried out in triplicate at 0, 4, 8, 24 and 48 h of fermentation only for the fruit juice with the best performance in bacteria growth during fermentation and viability in cold storage, i.e. apple juice. The superoxide anion scavenging activity of the fermented juices was determined by the WST (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2*H*-tetrazolium, monosodium salt) reduction method, using the SOD assay kit (Sigma-Aldrich, St.

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Louis, USA). This method is based on the reduction of WST-1 by superoxide radical (O_2•−) producing yellow formazan, which was measured at 450 nm in a spectrofluorometer SAFAS FLX-Xenius, Monaco. Antioxidant enzymes such as SOD inhibit the formation of WST-1. Before determination of the SOD activity, samples (1 ml) maintained at 5 °C were distributed into Eppendorf tubes and sonicated in an ultrasound bath (Fisher Bioblock Scientific, Illkirch, Belgium) at 50 W for 3 cycles of 2 min intercalated with 30 s of ice bath. The resulting suspension of bacteria cell lysate was then centrifuged at 3000 × *g* for 5 min. The supernatants were used for the SOD activity assay. SOD activity in the fruit juices was obtained from a standard curve calculated as equivalents SOD units/mg protein.

2.6. Evaluation of folates production

The determination of folate production by the lactobacilli strains was performed in triplicate in samples of 24 and 48 h of fermentation, for the fruit juice with the best bacteria growth and viability. In order to evaluate the influence of the addition of a precursor on the production of folates, *p*-aminobenzoic acid (PABA) was added at 10 μM into fruit juice before fermentation. Then the juice containing PABA was fermented only by the lactobacilli strains which produced folates in the preliminary evaluation. Fermented (8, 24 and 48 h of fermentation) and control juices were weighted (4 g), centrifuged at 3000 × *g* for 5 min, and the supernatant was taken for folate extraction, deconjugation, derivatization, purification and quantification as described by Delchier, Reich, and Renard (2012), adapted from Ndaw, Bergaentzlé, Aoudé-Werner, Lahély, and Hasselmann (2001), with a deconjugation step using pancreatic chicken homogenate (Pel-Freez Biologicals, Rodgers, AR, USA) at 5 g/l.

Quantification of folate was carried out on an HPLC (LC 20AD, Shimadzu Inc., Kyoto, Japan) equipped with fluorimetric detector recording at 295 nm and 356 nm of excitation and emission wavelengths, respectively. A reversed phase column LiChrospher 100RP18 (250 × 4.6 mm; 5 mm; Alltech, Epernon, France) and a guard column LiChrospher RP18 All Guard (7.5 × 4.6 mm; Alltech) were used for all analysis.

The volume of injection was 100 μl and the flow rate of 0.8 ml/min was applied to the mobile phase consisting of a gradient of solvent A: water and formic acid (10 ml/l) and solvent B: acetonitrile. Minor modification was made in the gradient of solvent, which started with 5% solvent B, increased linearly to 100% of solvent B in 20 min, then the gradient was held to 100% of solvent B for 10 min followed by linear decrease to 5% of solvent B in 5 min and an additional elution step for 5 min in order to re-equilibrate the column. Quantification of folates was done against calibration curves constructed with standards (Schircks laboratories, Jona, Switzerland): 5-methyltetrahydrofolic acid monoglutamate and 5-methyltetrahydrofolic acid diglutamate. Results are expressed in equivalent of 5-methyltetrahydrofolic acid monoglutamate in μg/100 ml of juice.

2.7. Hedonic analysis

The fermented juices with the best bacteria growth and viability, namely apple juices fermented by *L. acidophilus* L10, *L. casei* L26, *L. plantarum* 299v or *L. rhamnosus* 53103, were selected for the hedonic analysis. The 4 samples were tested by 29 untrained panelists formed by members of the research institute, who are used to analyze hedonic characteristics of food products. The samples were identified with random three-digit codes, distributed in portions of 30 ml into white cups and presented in randomized order. The panelists received the instruction to cleanse their palates with crackers and potable water between tasting samples, which were served at 5 °C ± 2 °C. In order to stave off the shortcoming tendency to avoid the use of the extreme of the categories (Jaeger & Cardello, 2009), the panelist was asked to mark her (his) evaluation about overall acceptability, odor, color and appearance on a 9 cm unstructured scale labeled with dislike extremely in the left and like extremely in the right end. The evaluations were measured with a rule and then converted into hedonic scale (between 0 and 1 cm = dislike extremely, 1–3 = dislike moderately, 3–5 = neither like nor dislike, 5–7 = like moderately, and 7–9 = like extremely), adapted from Peryam, Polemis, Kamen, Eindhoven, and Pilgrim (1960) and Caminiti et al. (2012). Participants were further asked to express their free comments on the juice. No information about the potential benefits of the fermented juices to health was given to the participants.

The microbiological safety at food degree of the samples was guaranteed according to European Legislation, through inspection for yeasts (<10/ml, method NF V08-059) and molds (<10/ml, method NF V08-059), total coliforms (<10/ml, method Petrifilm coliforms 3 M® – 01/2–09/89 A), *Escherichia coli* β glucuronidase positive (<10/ml, method Petrifilm E. coli 3 M® – 01/8–06/01), *Staphylococcus aureus* (<10/ml, method NF V08-057), *Pseudomonas* sp. (<10/ml, method NF EN ISO 13720), *Salmonella* sp. (absence/25 g, method IBISA-AES 10/11–07/11), *Listeria monocytogenes* (<100/g, method NF EN ISO 11290-2) and *Bacillus cereus* (<100/ml, method NF EN ISO 7932) by an independent certified laboratory (Laboratoire Départemental d'Analyses, Vaucluse, Avignon, France). Hedonic analysis took place 5 days after the fermentation, when the certificate of microbiological safety was delivered. Until then, samples were maintained in refrigerated storage at 5 °C.

2.8. Statistical analysis

Data were analyzed by means of ANOVA using XLSTAT® (Addinsoft SARL) data analysis toolbox. Mean differences amongst treatments were assessed by the post-hoc Tukey test considering $P < 0.05$ as significant level. General Linear Model was applied to evaluate the effect of treatment (fermentation or supplementation) and lactobacilli counts during the cold storage period. Statistically significant differences were indicated by labeling the mean values with different letters.

3. Results and discussion

3.1. Lactobacillus counts and pH during fermentation of apple, grape and orange juices

Taken as a whole, there was a significant effect of juice type on lactobacilli growth by 48 h of fermentation, being their average counts higher in apple juice (8.7–10.3 log CFU/ml) than in grape (8.0–9.8 log CFU/ml) and orange (7.9–8.4 log CFU/ml) juices ($P < 0.05$), Fig. 1. Furthermore, commercial lactobacilli strains adapted faster to apple juice – in 8 h of fermentation the bacteria counts increased about 2 log CFU/ml – and reached higher counts than the lactobacilli of the same species but isolated from artisanal products (Fig. 1). Noteworthy, *L. casei* L26 was the strain with the highest counts at 48 h of fermentation of grape juice. In all fruit juices, *L. rhamnosus* strains LGG and 607 reached maximum counts at 48 h of fermentation (Fig. 1).

The initial pHs (pH at t0) of apple, grape and orange juices were 3.7 (±0.1), 3.4 (±0.1) and 3.6 (±0.1), respectively. The average pH increased by 0.1 units in the first 4 h of fermentation of apple and grape juices (Fig. 2). As malic acid is the main organic acid in apple and grape juices – but not in orange juice – (Del Campo, Berregi, Caracena, & Santos, 2006), the period of de-acidification observed at the early stages of fermentation can be mostly ascribed to the conversion of malic acid (a dicarboxylic acid), into lactic acid (a weaker monocarboxylic acid) and carbon dioxide (Liu, 2002; Toit, Engelbrecht, Lerm, & Krieger-Weber, 2011) by most of the *Lactobacillus* strains tested herein.

The averages of pH at 48 h of fermentation were strain-dependent and ranged between 3.4–3.6, 3.3–3.5 and 3.2–3.4 in apple, grape and orange juices, respectively (Fig. 2).

As expected, lactobacilli counts were inversely and strongly correlated to pH ($r = -0.729$, $P < 0.01$) in all fruit juices. As a matter of fact, a low pH was indicated by many authors as one of the main growth-limiting factors for lactic acid bacteria (Serrazanetti, Guerzoni, Corsetti,

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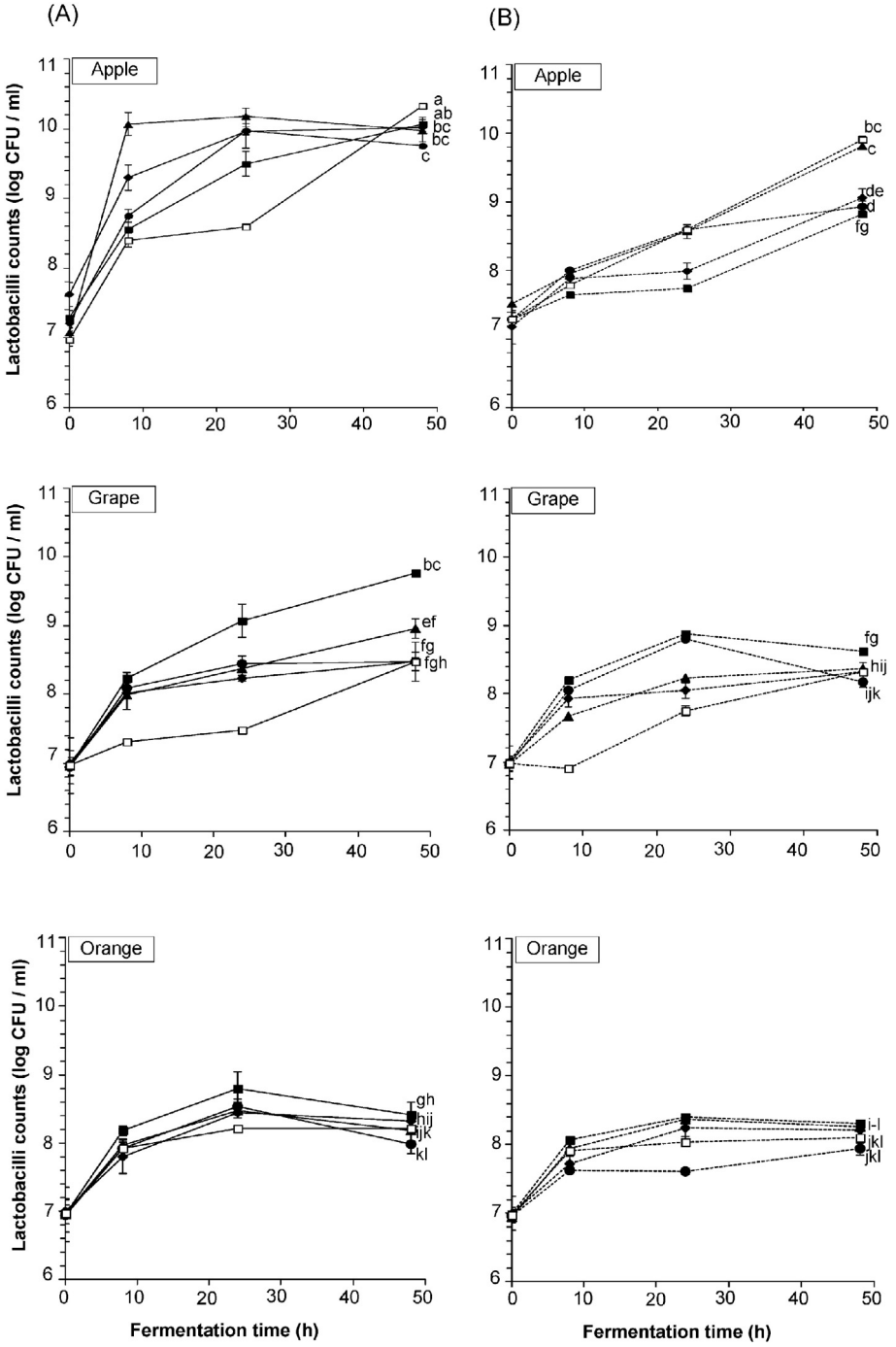

Fig. 1. Bacteria counts during 48 h of fermentation at 40 °C of apple, grape and orange juices fermented by Lactobacilli strains from (A) commercial and (B) artisanal products. Different letters indicate significant differences ($P < 0.05$) in the mean bacteria counts for each strain–juice combination after 48 h fermentation. Bars represent standard deviation of triplicate measurements of duplicate fermentations. —◆— *L. acidophillus* L10, —■— *L. casei* L26, —▲— *L. paracasei* L33, —●— *L. plantarum* 299v, —□— *L. rhamnosus* LGG ATCC 53103, ---◆--- *L. acidophillus* CIRMBIA 1674, ---■--- *L. casei* CIRMBIA 667, ---▲--- *L. paracasei* CIRMBIA 672, ---●--- *L. plantarum* CIRMBIA 466, and ---□--- *L. rhamnosus* CIRMBIA 607.

& Vogel, 2009; Yanez, Marques, Girio, & Roseiro, 2008). However, as also reported by Sheehan, Ross, and Fitzgerald (2007), the variations of bacteria growth in different fruit juices cannot be ascribed only to the limited variations in pH, but also to the juices chemical composition, as a balance between nutrient and inhibitor compounds for lactobacilli and to the capacity of the strains to adapt to these stressful matrices.

In spite of the former low pH in fruit juices and the highly probable presence of inhibitor compounds, *Lactobacillus* strains were able to grow and ferment the fruit juices tested. Rakin et al. (2007) subjected beetroot and carrot juices enriched with brewer's yeast autolysate to fermentation with *L. acidophilus* NCDO1748, obtaining a bacteria growth of about 1–2 log CFU/ml by the end of the process, which is similar to the results obtained in the present study for fruit juices without additives.

3.2. Lactobacillus counts and pH after cold storage of fermented and of supplemented juices

Based on the evidence that apple juice was the best substrate for all the lactobacilli strains tested, further analyses were done only for this fruit juice.

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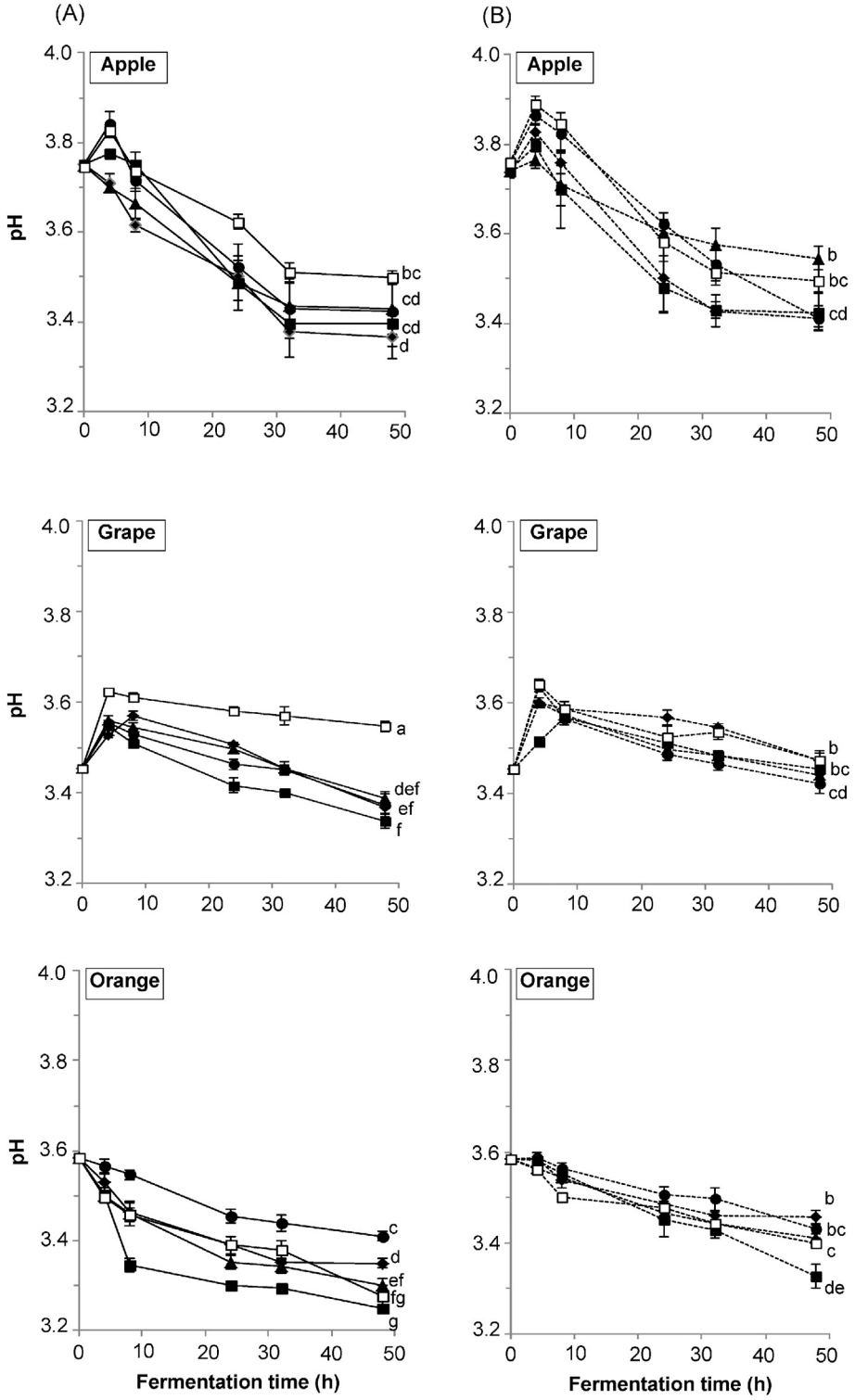

Fig. 2. Changes in pH during 48 h of fermentation at 40 °C of apple, grape and orange juices by Lactobacilli strains from (A) commercial and (B) artisanal product origin. Different letters indicate significant differences ($P < 0.05$) in the mean bacteria counts for each strain–juice combination after 48 h of fermentation. Bars represent standard deviation of triplicate measurements of duplicate fermentations. —◆— *L. acidophillus* L10, —■— *L. casei* L26, —▲— *L. paracasei* L33, —●— *L. plantarum* 299v, —□— *L. rhamnosus* LGG ATCC 53103, ---◆--- *L. acidophillus* CIRMBIA 1674, ---■--- *L. casei* CIRMBIA 667, ---▲--- *L. paracasei* CIRMBIA 672, ---●--- *L. plantarum* CIRMBIA 466, and ---□--- *L. rhamnosus* CIRMBIA 607.

Table 1 presents the decrease of lactobacilli viability calculated as the difference between the bacteria counts on days 1 and 28, and the pH of the apple juices on day 28.

Considering the whole period of cold storage, apple juices fermented by *L. acidophilus* L10 strains and 1674, *L. casei* strains L26 and 667, *L. paracasei* 672, *L. plantarum* 299v and *L. rhamnosus* LGG, showed higher counts of lactobacilli than in juices supplemented with the same strains ($P < 0.01$). Moreover, in average, lactobacilli from commercial products had higher cell viability during 28 days than those isolated from artisanal products. The decrease in lactobacilli counts ranged from 0.6 to 4.0 log CFU/ml, and from 3.3 to 4.8 log CFU/ml in fermented and supplemented juices, respectively, being the lowest in apple juice fermented by *L. rhamnosus* LGG ($P < 0.01$), and always below the decrease of 7 log CFU/ml during cold storage observed by Ding and Shah (2008) in

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Table 1
Decrease of Lactobacilli counts and pH on day 28 of cold storage of supplemented and fermented apple juices.

Microorganisms	Apple juice	ΔLactobacilli counts (log CFU/mL)[a,*]	pH on day 28[**]
Control juice	Not inoculated		3.75 ± 0.03 a
L. acidophilus L10	Supplemented	3.59 ± 0.03 bc	3.70 ± 0.05 abc
L. casei L26	Supplemented	3.27 ± 0.01 cde	3.68 ± 0.02 bc
L. paracasei L33	Supplemented	3.32 ± 0.01 cd	3.67 ± 0.03 bc
L. plantarum 299v	Supplemented	3.35 ± 0.01 cd	3.70 ± 0.02 abc
L. rhamnosus LGG	Supplemented	3.39 ± 0.01 cd	3.65 ± 0.03 ab
L. acidophilus 1674	Supplemented	4.61 ± 0.02 a	3.72 ± 0.02 abc
L. casei 667	Supplemented	4.77 ± 0.02 a	3.72 ± 0.02 abc
L. paracasei 672	Supplemented	4.69 ± 0.01 a	3.70 ± 0.03 abc
L. plantarum 466	Supplemented	3.36 ± 0.01 cd	3.70 ± 0.02 abc
L. rhamnosus 607	Supplemented	3.92 ± 0.26 b	3.69 ± 0.03 bc
L. acidophilus L10	Fermented	2.01 ± 0.05 gh	3.30 ± 0.02 def
L. casei L26	Fermented	2.20 ± 0.01 g	3.35 ± 0.04 def
L. paracasei L33	Fermented	2.94 ± 0.10 de	3.25 ± 0.04 g
L. plantarum 299v	Fermented	1.66 ± 0.09 h	3.35 ± 0.03 def
L. rhamnosus LGG	Fermented	0.64 ± 0.01 i	3.41 ± 0.04 de
L. acidophilus 1674	Fermented	2.36 ± 0.02 fg	3.36 ± 0.05 def
L. casei 667	Fermented	2.77 ± 0.01 ef	3.38 ± 0.04 def
L. paracasei 672	Fermented	3.76 ± 0.03 bc	3.49 ± 0.03 d
L. plantarum 466	Fermented	3.55 ± 0.05 bc	3.37 ± 0.03 def
L. rhamnosus 607	Fermented	4.01 ± 0.06 b	3.44 ± 0.04 de

[a] Δ*Lactobacillus* counts = *Lactobacillus* counts on day 1 − Lactobacillus counts on day 28 (log CFU/mL).
* Values given are means (n = 6) ± sum of the variances. Means with different letters in the same column are significantly different (P < 0.05).
** Values given are means (n = 6) ± standard deviation. Means with different letters in the same column are significantly different (P < 0.05).

buffered apple juice supplemented with free *L. acidophilus*, *L. paracasei*, *L. plantarum* or *L. rhamnosus*. The viability of *Lactobacillus* during 4 weeks of cold storage in the apple juice tested herein was higher than in pomegranate juice fermented by *L. plantarum*, *L. acidophilus* and *L. paracasei* (Mousavi, Mousavi, Razavi, Emam-Djomeh, & Kiani, 2011) and comparable to the probiotic bacteria viability in stirred fruit yoghurts (Kailasapathy, Harmstorf, & Phillips, 2008) and even in petit Suisse cheese (Esmerino et al., 2013). Apple juice thus appeared to be a suitable matrix to insure the viability of lactobacilli during cold storage. A possible explanation for the better lactobacilli viability in supplemented apple juice in our study could be the gradual adaptation of the cultures to the stressful conditions of the fruit juices, as described in Section 2.3.

Noteworthy, during 4 weeks of cold storage, lactobacilli counts remained above 5–6 log CFU/ml which represents, considering a dose of 100 ml of fermented apple juice, the generally recommended daily probiotic intake of 8 log CFU to be perceived beneficial effects to the host (Mihatsch et al., 2012; Taverniti, Scabiosi, Arioli, Mora, & Guglielmetti, 2014).

Various methods have been proposed to improve cell viability during storage: microencapsulation of probiotic bacteria (Burgain, Gaiani, Linder, & Scher, 2011; Ding & Shah, 2008; Nualkaekul, Lenton, Cook, Khutoryanskiy, & Charalampopoulos, 2012; Rokka & Rantamäki, 2010), adhesion to prebiotic fibers (Saarela, Virkajärvi, Nohynek, Vaari, & Mättö, 2006) or addition of protective compounds and pH control of the food matrix (Pereira, Maciel, & Rodrigues, 2011; Rakin et al., 2007). Herein, the best survival of lactobacilli during cold storage of fermented apple juice might be due to the selection, during fermentation, of bacteria individuals that mostly adapted to the apple juice environment, thus surviving longer than the same *Lactobacillus* strains simply supplemented into apple juice. This observation encourages the fermentation of apple juice as an alternative to maintain the recommended beneficial bacteria counts during the shelf-life.

During cold storage, the pH of the lactobacilli-supplemented apple juices did not decrease significantly in relation to the non-supplemented control (P > 0.05), Table 1. Likewise, the pH of the fermented apple juices showed no difference (P > 0.05) between the days 1 and 28 of cold storage, which could be ascribed to the reduction on cell viability with consequent reduction of organic acid production, Table 1.

The decrease of pHs (0.4 unit maximum) during fermentation and cold storage, could be regarded as the result of different acidifying capacity of the strains in the juices tested, and also to the buffering capacity of these substrates (Lussi, Kohler, Zero, Schaffner, & Megert, 2000).

3.3. Superoxide dismutase activity

SOD activity in control apple juice (non-inoculated) at t0 was of 9.9 units/mg protein, close to the 10 units/mg protein reported by Masia (1998) in Golden apple extracts, considering the dry weight. Nevertheless, this initial SOD activity was significantly reduced to 5.8 units/mg protein at t48 (P < 0.01), Fig. 3A and B.

SOD activity decreased sharply yet in the first 4 h of fermentation of apple juice by most of the lactobacilli strains — particularly those from commercial origin, Fig. 3A and B. The reduction of SOD activity in control juice and in fermented juice at the early stages of fermentation can be due to the sensibility of the enzyme to the temperature of fermentation (40 °C), its inactivation by compounds present in the fruit juice and/or produced by the lactobacilli such as exopolysaccharides as a reaction to acidic stressful environment (Welman & Maddox, 2003), or by a combination of factors. Further studies are needed to elucidate what these SOD-inhibitors are.

On the other hand, the SOD activity increased significantly after 4 h of fermentation and its maximum ranged from 15.3 to 15.9 and from 9.2 to 14.0 units/mg of protein in apple juices fermented by strains from commercial and from artisanal origin, respectively (Fig. 3A and B). Furthermore, in general, the maximum SOD activity was reached in 24 h of fermentation by commercial strains of *Lactobacillus* and in 48 h in apple juices fermented by strains from artisanal product origin. Taken as a whole, the SOD activity in all fermented apple juices was found to be strongly correlated to bacteria counts (r = 0.624, P < 0.001), pointing out that the ability of lactobacilli to produce anti-oxidative substances such as SOD is one of the factors of furthest importance to cell viability, as they minimize the deleterious effects of ROS accumulated during fermentation in aerobic conditions (Lin & Yen, 1999). To the best of our knowledge, it is the first time that the profile of production of SOD by lactobacilli during fermentation of a fruit juice is reported.

3.4. Production of folates

The presence of folates – expressed as folic acid equivalent – was observed only in apple juice fermented by strains of *L. plantarum* and *L. rhamnosus*. As these *Lactobacillus* species are also consumers of folates, the averages of folic acid equivalent presented in Fig. 4A and B should be taken as the result of folates production and consumption.

On the one hand, folate production began at 24 h of fermentation but the highest concentrations were reached at 48 h in juices fermented by the commercial strains *L. plantarum* 299v (1.03 μg/100 ml) and *L. rhamnosus* LGG (1.26 μg/100 ml). On the other hand, when PABA was added at 10 μM, the production of folates was higher at 24 h than at 48 h of fermentation (P < 0.05) and the concentrations were the highest in apple juices fermented by the two strains of *L. plantarum*, Fig. 4A and B. Considering the strains of *L. plantarum* 299v and CIRMBIA 466 and of *L. rhamnosus* LGG and CIRMBIA 607, the folates level was positively correlated with *L. plantarum* and *L. rhamnosus* counts (r = 0.627, P < 0.001) either supplemented or not with PABA. The supplementation of apple juice with PABA may have anticipated by 24 h the peak of maximum lactobacilli counts (data not shown), and thus the formation of folates. However production of folates was not increased in proportion to the amount of precursor added (10 μM), as maximum folate concentration in the medium was below 1.8 μg/100 ml (0.4 μM). The influence of bacteria counts and fermentation time on folate level was also reported by Lin and Young (2000), who observed a significant reduction in folate

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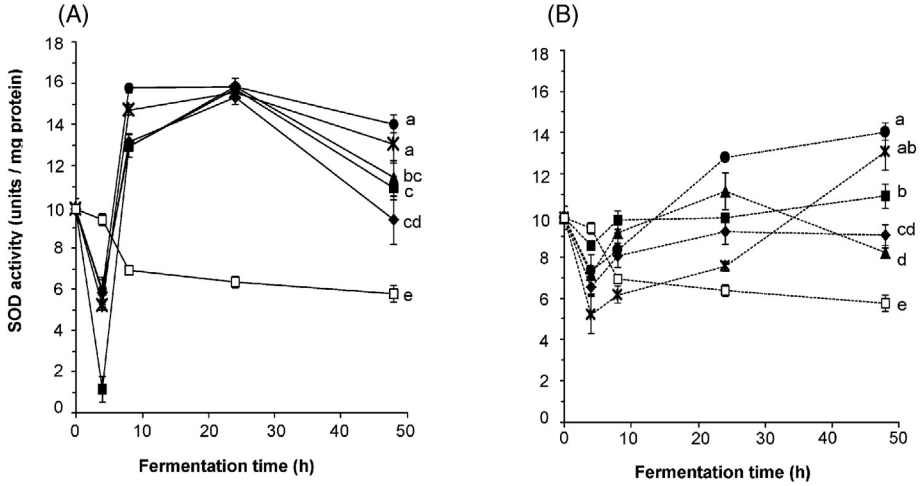

Fig. 3. Superoxide dismutase activity (inhibition %) during fermentation of apple juice by (A) commercial Lactobacilli strains and (B) Lactobacilli strains isolated from artisanal products. Apple juice without inoculum and maintained under the same conditions of the fermentation was used as control. Means of each one of the 11 treatments, with different letters are significantly different ($P < 0.05$); $n = 3$. Bars represent standard deviation. —◆— *L. acidophillus* L10, —■— *L. casei* L26, —▲— *L. paracasei* L33, —●— *L. plantarum* 299v, —□— *L. rhamnosus* LGG ATCC 53103, ---◆--- *L. acidophillus* CIRMBIA 1674, ---■--- *L. casei* CIRMBIA 667, ---▲--- *L. paracasei* CIRMBIA 672, ---●--- *L. plantarum* CIRMBIA 466, and ---□--- *L. rhamnosus* CIRMBIA 607.

concentration after this reached a maximum during fermentation of complex media by *Lactobacillus* strains.

The amounts of folates present in apple juices fermented by *L. plantarum* and *L. rhamnosus* strains are not high enough to consider them as source of folates, which recommended dietary allowances (RDA) range between 300 μg–1000 μg, depending on population category (Morales, Fernández-Ruiz, Sánchez-Mata, Cámara, & Tardío, 2015). Notwithstanding, these findings encourage the pursuit of prospective studies for strain selection in order to increase folates production by *L. plantarum* and *L. rhamnosus* in vegetable food matrix.

3.5. Hedonic analysis

Participants ($n = 29$) in the panel were mostly women (62%), 26–35 years old (28%) or 36–50 years old (34%), pay attention to their daily diet (55%), drink fruit juice 1–5 times/week (55%) and are familiar with fermented foods (100%).

Although the average scores for overall acceptability, taste and appearance were not significantly different (5.1–6.2 = like moderately), a tendency of higher scores for apple juices fermented by *L. acidophilus* L10 was observed, as summarized in Fig. 5. However, the odor of the apple juice fermented by *L. rhamnosus* 53103 was significantly ($P < 0.05$) less appreciated (3–5 = neither like nor dislike) than the juice fermented by *L. acidophilus* L10 (6–7 = like moderately). Considering the average score in the hedonic scale for overall acceptability, apple juice fermented by *L. plantarum* 299v received the worst acceptance (17% dislike extremely) and those fermented by *L. acidophilus* L10 and by *L. casei* L26 were the best accepted, with 28 and 24%, respectively, of scores like very much–like extremely (Fig. 5).

Most of the participants who left further observations, considered the taste "characteristic of apple juice" (19) and "sweet" (17). Only 3 participants remarked a "baked apple" taste in the samples, which can be ascribed to the exposition of the juices to 40 °C during 48 h of fermentation. A "fermented dairy" taste and odor were perceived by 10 participants, as the result of fermentation by lactic acid bacteria. The color was described as "dark yellow" (2), "bright yellow" (3), "correct for an apple juice" (3) and "very pale yellow" (9) in apple juices fermented by *L. acidophilus* L10, *L. casei* L26, *L. plantarum* 299v and *L. rhamnosus* LGG ATCC 53103, respectively.

The taste and odor of fermented dairy food can be probably ascribed to the presence of compounds such as acetoin, diacetyl and 2,3-butanediol produced by LAB from the catabolism of pyruvate under

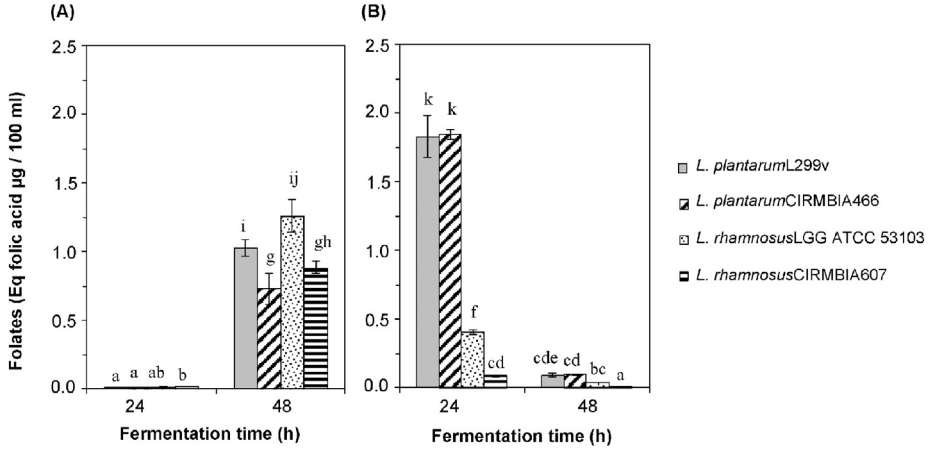

Fig. 4. Folates concentration (equivalent in folic acid μg/100 ml) in apple juice without PABA (A) and with 10 μM of PABA (B) fermented by *Lactobacillus plantarum* strains 299v and CIRMBIA 466 and *L. rhamnosus* strains LGG ATCC 53103 and CIRMBIA 607. Means with different letters are significantly different ($P < 0.05$), $n = 3$.

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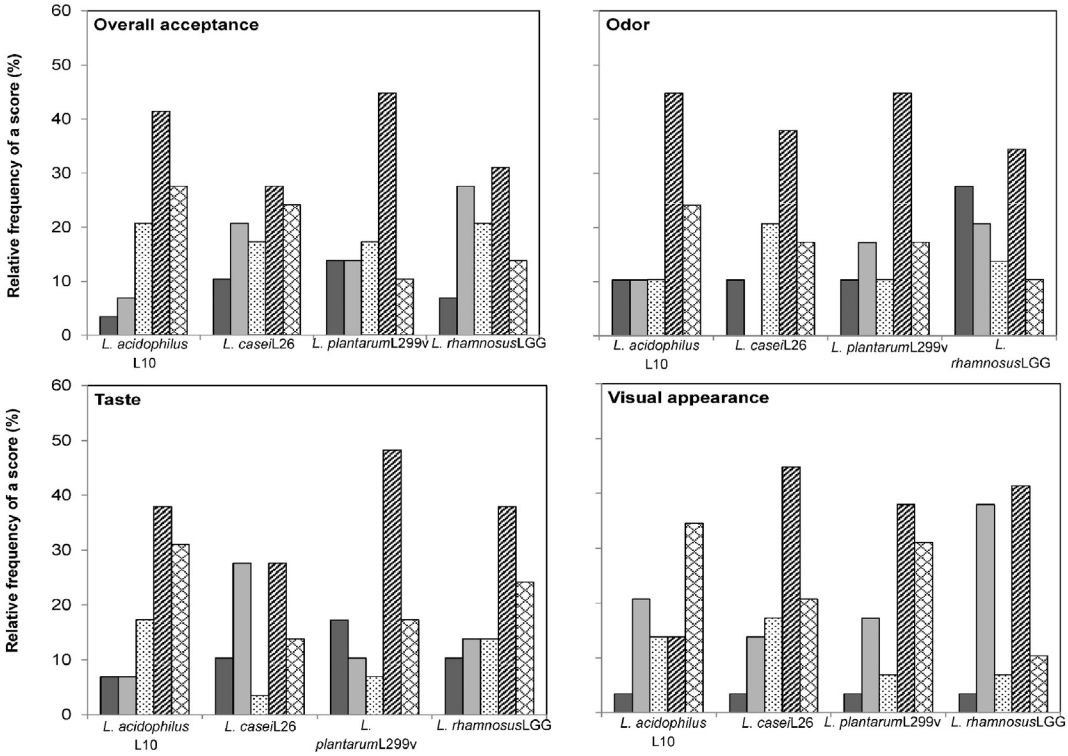

Fig. 5. Distribution of scores for the hedonic parameters of apple juices fermented by *L. acidophillus* L10, *L. casei* L26, *L. plantarum* 299v, and *L. rhamnosus* LGG ATCC 53103; n = 29. Hedonic scale: 0–1 = Dislike extremely; 1–3 = Dislike moderately; 3–5 = Neither like nor dislike; 5–7 = Like moderately; 7–9 = Like extremely.

aerobic conditions (Liu, 2003). As the lactobacilli consumed sugars present in the apple juice during fermentation (data not shown), the sweetness remarked by some of the participants can be due to the apple variety chosen for this study, i.e. *var.* Golden, which has a high sugar/acid ratio (Eisele & Drake, 2005). Furthermore, the conversion of malic into the weaker lactic acid (Toit et al., 2011) by *Lactobacillus* may have reinforced the sweet in-mouth feeling.

The overall acceptability was found to be strongly correlated with the taste ($r = 0.845$, $P < 0.0001$) and with the odor ($r = 0.713$, $P < 0.0001$), but not with the visual appearance ($P > 0.05$) of the fermented juices.

The flavors of orange juices supplemented with *L. rhamnosus* LGG, *L. casei* Imunitass®, or *L. paracasei* NFBC 43338 are described as 'dairy,' 'savory,' and 'medicinal' (Luckow, Sheehan, Delahunty, & Fitzgerald, 2005). However, after 7 days of consumption of probiotic supplemented juices, a 'mere-exposure' effect – which is the increase in liking scores upon frequent consumer exposure to a food product – is observed (Luckow et al., 2005). Thus, to improve the acceptation of apple juices fermented by Lactobacilli, one can suggest the familiarization of the consumers to this new product.

4. Conclusions

In general, lactobacilli counts were higher in apple juice than in grape and orange juices by 48 h of fermentation. Moreover, the commercial lactobacilli strains *L. acidophilus* Lafti L10, *L. casei* Lafti L26, *L. plantarum* 299v and *L. rhamnosus* LGG ATCC 53103 showed higher growth and viability in fermented apple juice than the ones isolated from artisanal products: *L. acidophilus* CIRMBIA 1674, CNRZ 204, *L. casei* CIRMBIA 667, CNRZ 313, *L. plantarum* CIRMBIA 466, CNRZ 211 and *L. rhamnosus* CIRMBIA 607, CNRZ 212, which highlights the importance of prospective studies for selection of the strain the most adaptable to a given food substrate.

The fermentation process, rather than the simple supplementation, insured greater viability of most of the lactobacilli strains tested during the four weeks of cold storage. Furthermore, other benefits of fermentation were observed such as an increase of SOD activity and folates concentration in apple juice. To the best of our knowledge, it is the first time that the production of folates in apple juices fermented by *L. plantarum* and by *L. rhamnosus*, is reported.

In spite of the fermented dairy odor remarked by some members of the panel, the overall acceptability, odor, taste and visual appearance of apple juices fermented by *L. acidophilus* Lafti L10, *L. casei* Lafti L26, *L. plantarum* 299v and *L. rhamnosus* LGG ATCC 53103 were scored as "like moderately", encouraging further studies of product development.

These findings meet the needs of the expanding probiotic market, eager for novelties, suggesting apple juice as a suitable alternative to the dairy-based matrices for probiotic food formulation and fermentation as a process to improve the *Lactobacillus* viability and potentially, the functional aspects of the food product.

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