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Development of a potentially probiotic food through fermentation of Andean tubers

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ABSTRACT

With the aim to obtain a non-dairy solid probiotic product, three different Andean tubers -oca (*Oxalis tuberosa)*, papalisa (*Ullucus tuberosus)* and potato (*Solanum tuberosum* spp andigena)- were assessed as fermentation substrates for the potentially probiotic *L. brevis* CJ25 strain. Fermented Churqueña potato puree, oregano and NaCl were used for the manufacture of a product called Potato Cheese due to its firm texture. This functional food contains a viable cell concentration of 8.0 log CFU/g and pH 5.1. Even after 28 days of storage at 4 °C, increases in cell counts were found and pH decreased 0.7 units, which improved food safety avoiding the growth of spoilage and pathogenic microorganisms. The *in vitro* tests indicated that *L. brevis* CJ25 exhibited high level of survival in simulated gastric juice (3 h, pH 2.5) when delivered in Potato Cheese, and also showed resistance to bile salt after 3 h of exposure. These results suggest that Potato Cheese is a promising novel functional food with high nutritional value, free of cholesterol and lactose and its development may have a favorable impact in Andean regional economies.

1. Introduction

In the Andean region of Argentina there is a wide diversity of crops, among these, potato (*Solanum tuberosum* spp *andigenum*), oca (*Oxalis tuberosa*) and papalisa (*Ullucus tuberosus*) represent an alternative to cover increasing demands in human alimentation. These tubers show an extensive variety of shapes and colors and offer a wide antioxidant profile (Condori et al., 2008). Research on local and ancient crops and their use to develop new products have a worldwide renewed interest (Coda, Cagno, Gobbetti, & Rizzello, 2014) and may represent an opportunity to enhance the regional economies.

Lactic acid bacteria (LAB) are the group most widely used in the fermented food industry since they improve nutritional, technological and sensorial characteristics and play a protective role against spoilage and pathogen microorganisms by lowering pH, competing for nutrients and producing antimicrobial compounds such as organic acids, H_2O_2, diacetyl and bacteriocins in some cases (Ramos, Thorsen, Schwan, & Jespersen, 2013; Swain, Anandharaj, Ray, & Praveen Rani, 2014). Foods containing probiotics fall within the category of functional foods, which are claimed to have a positive effect on health since these bacteria reach the intestine where they exert a number of benefic effects (Govender et al., 2014; Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013). Consequently, candidate probiotics must be able to survive through gastrointestinal (GI) simulated conditions which include *in vitro* tests to assess the tolerance to low pH and bile salts and finally *in vivo* and clinical studies to validate the functional properties (Saarela, Virkajarvi, & Alakomi, 2006). The number of viable microorganisms at the time of consumption is extremely important to provide expected health benefits. Despite the effective dose may be determined for each particular case, many authors have suggested a minimum dose between log 6.0 and log 9.0 CFU/g to assure a beneficial effect (Kim, Jang, & Yoon, 2012; Silva, Bezerra, Santos, & Correia, 2015).

Food matrix is an important factor in the development of a probiotic food. The effect of additives or spices in the bacterial growth has been studied since some authors suggested that the substrate composition could affect growth and survival during fermentation, processing and storage, and even the stability of the probiotic microorganisms throughout GI transit may be enhanced by some food components (Charalampopulos, Pandiella, & Webb, 2003; Do Espírito Santo, Perego, Converti, & Oliveira, 2011). Particularly, it was reported that some spices have positive influences in the growth of several LAB strains (Marhamatizadeh

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et al., 2012; Zaika & Kissinger, 1981).

Although dairy and meat products are the most common fermented food, some particular vegetables are fermented as traditional practices in different cultures (e.g. sauerkraut, miso, soy sauce, pickled vegetables and kimchi) (Swain et al., 2014). Nowadays it is important to offer non-dairy probiotic products to cover the increasing demand of persons with lactose intolerance, high cholesterol or vegetarians (Kim et al., 2012). Fruit, vegetables and cereals are considered suitable matrices for probiotic foods because their nutrients are easily assimilated by bacteria (Martins et al., 2013). In this sense, some of them such as beetroot, apple, pear, soybean, carrot and tomato, among others, were successfully employed to design drinks as probiotic carriers since LAB were capable to produce high amounts of lactic acid and maintain desirable cell counts during shelf-life (Alegre, Viñas, Usall, Anguera, & Abadias, 2011).

The aim of this study was to determine the suitability of different Andean tubers as fermentation substrates for a potentially probiotic strain of *Lactobacillus brevis* and the development and characterization of a solid probiotic product.

2. Materials and methods

2.1. Fermentation substrates

Three varieties of Andean tubers were studied: Churqueña potato (CH) (*S. tuberosum* spp andigena), Oca morada (OC) (*O. tuberosa*) and Papalisa rosada (PL) (*U. tuberosus*). Tubers were purchased from local producers in a rural cooperative from Quebrada de Humahuaca (Jujuy, Argentina) and stored in a cold chamber prior to use. The Spunta (SP) (*S. tuberosum*) variety was procured in a local market and used as a reference because it is the most widespread commercially. Tubers were washed and cooked in water for 20 min. Unpeeled tubers were mashed with a commercial food processor to prepare the purees.

2.2. Strain and fermentation procedure

The microorganism used in this study is part of the Jujuy National University collection and was previously isolated from a goat's cheese of the Andean region. It was identified as *L. brevis* CJ25 using 16S rRNA gene sequencing. Genomic DNA was used for amplification using the primers 616 Valt and 630R (Chenoll, Macián, Elizaquivel, & Aznar, 2007). The resulting PCR products were purified using the NucleoSpin Extract II Kit (Macherey–Nagel, Düren, Germany) and sequenced using the ABI-PRISM 377 (PE-Applied Biosystems, Foster City, CA, U.S.A.) automated sequencer and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE-Applied Biosystems, Foster City, CA, U.S.A.). The 16S rDNA sequence was compared with the NCBI database using the Sequence Comparison BLAST tool (http//:www.ncbi.nlm.nih.gov) showing sequence similarity level of 99%. The strain was subcultured twice for 12 h at 32 °C in MRS broth (Britania Co., Buenos Aires, Argentina) and used to inoculate (1.5% v/w; initial cell number corresponding to ca. 6.0 log CFU/g of puree) each of the purees, under strictly sterile conditions. The fermentations were carried out for 72 h at 32 °C in vacuum sealed plastic bags containing 50 g of each puree. Unstarted purees were used as controls.

2.3. Chemical, physical and microbiological analyses

Samples y controls were taken at 24 h intervals for physical and chemical analyses. pH, total titratable acidity (TTA) and total reducing sugars (TRS) were performed in solutions containing 10 g of each puree and 90 ml of distilled water. TTA was determined tritating with 0.1 M NaOH and expressed as g lactic acid/g puree. The values of pH were measured using a digital pH meter (DALVO, MHS 400). To determine TRS the 3.5-Dinitrosalicilic acid (DNS) method was used (Miller, 1959) and results were expressed as g glucose/g puree. To measure the buffering capacities, 100 ml of each media was titrated with HCl (Pai, Tsau, & Yang, 2001) and the values were expressed as the amount of HCl (mmol) needed to drop 1 pH unit per unit volume (1 L). Viable cell counts (log CFU/ml) were also determined at 8 and 16 h of fermentation, by the standard plate method with MRS agar after 48 h of incubation at 32 °C.

2.4. Manufacture and characterization of a probiotic product

CH potato was selected to develop a solid potentially probiotic product. 1.5% NaCl and 0.8% oregano were added to improve organoleptic characteristics. Proximate composition of products and raw materials was determined using AOAC (1998) methods: Moisture (964.22), samples were drained in a conventional oven at 105 °C to constant weight; Proteins (984.13), by Kjedahl method using 6.25 as the nitrogen-to-protein conversion factor; Ash (923.03), samples were burned in a muffle furnace at 550 °C to obtain white ash; Lipids (920.39), by Soxhlet technique. Total carbohydrates were calculated as difference [100 − (proteins + lipids + ash + moisture)].

Water activity was measured using a Water Activity Detector (Aqualab 3TE) at 25 °C. The effect of NaCl and oregano on total count was assessed by determining viable LAB cells in the puree with and without additives at 0, 8, 16, 24, 48 and 72 h of fermentation.

2.5. Effect of cold storage on cell counts

The product was stored 4 weeks at 4 °C and samples were collected weekly. In order to evaluate the growth of *L. brevis*, pH and viable cell counts were estimated. Enterobacteria content was estimated using Violet Red Bile Glucose Agar media (Britania) and incubated at 32 °C for 24 h. The number of yeasts was estimated on Sabouraud Dextrose Agar media (Britania), supplemented with chloramphenicol (0.1 g/L) at 30 °C for 48 h.

2.6. In vitro gastrointestinal tolerance assay

Tolerance to gastrointestinal conditions of *L. brevis* contained in the final product was studied *in vitro* with the technique described by Kim et al. (2012) with modifications.

Simulated GI juices were prepared with phosphate buffered saline (PBS) buffer solution, adjusted to different pH conditions.

The PBS pH was set at 2.5 and 6.4 (control) by the addition of 6 N HCl solution to simulate gastric stress. 0.3% (w/v) of bile salt (Oxoid, Hampshire, UK) was added to PBS and pH was adjusted to 8.0 with NaOH 1 M solution to mimic enteric conditions. Solutions were autoclaved at 121 °C for 15 min, brought to 32 °C and then inoculated with 10% (v/v) of the BAL contained in the product (5 g of fermented product were homogenized in 45 ml of distilled water) and incubated for 3 h at 32 °C. A combined treatment was carried out and included 3 h of gastric phase followed by 3 h of enteric phase. Tolerance to GI simulated conditions was determined by subsequent growth on MRS agar as described previously.

A control was prepared at investigating the intrinsic tolerance of *L. brevis* grown in MRS to GI conditions. LAB cells were cultivated in MRS broth at 32 °C for 12 h, centrifuged (5000 rpm for 5 min), washed 2 times in PBS (pH 6.4) and the assay was performed according to the method described above.

2.7. Statistic analysis

All experiments were conducted in triplicate and the results were expressed as mean ± SD (standard deviation). Statistical analyses were performed with the software Statistica 7.0 (StatSoft, Inc., USA) using ANOVA followed by a Tukey's test, and differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Physicochemical parameters

In order to compare the growth pattern of *L. brevis* CJ25 in different boiled tuber substrates, preliminary experiments included the evolution of cell populations, pH, TRS and TTA concentrations during fermentation. Results are shown in Fig. 1.

Cell number of *L. brevis* CJ25 increased rapidly in the four media at the beginning and reached the stationary pse within 16 h with cell populations above 10.0 log CFU/g which indicates that they are all suitable substrates. After 6 h of fermentation, viable cell counts were higher than the suggested minimum of 6.0 log CFU/g for a probiotic food (Peres, Peres, Hernández-Mendoza, & Malcata, 2012).

OC had an initial pH of ca. 4.2 ± 0.2, probably due to its content of oxalic acid (Espín, Villacrés, & Brito, 2003), it decreased to 3.8 ± 0.2 during the first 24 h and then remained stable. Initial pH values in CH and PL purees were ca. 6.0, decreased during the first 8 h and stayed stable until 72 h. Initial and final pH of SP did not differ significantly ($P < 0.05$) which could be related with the highest buffer capacity of this tuber (52 ± 2.8 mmol HCL $pH^{-1}\,l^{-1}$), twofold higher than OC, PL and CH (27 ± 1.2; 22 ± 0.9; 19 ± 0.8, respectively). de Willigen (1950) suggested that some mineral salts contained in chemical fertilizers increase buffer capacity. Initial concentrations of TRS in OC, PL and SP media were 2944 ± 183, 2496 ± 158 and 912 ± 73 mg glucose/100 g puree, respectively, and decreased significantly ($P < 0.05$) throughout fermentation. In contrast, reducing sugar content in CH potato remained unchanged. Notwithstanding this, the greatest increase in TTA was found for CH during the first 24 h, indicating that fermentation conditions were appropriate and this media provided all the required nutrients for growth and acid production. With the aim to obtain a solid functional food, PL and OC purees were not considered for further experiments due to they gave too liquid matrices with fast phase separation and difficulties in homogenization. Based on the above results, and since CH media determined the best sensory perception (data not shown), CH was used to manufacture a solid fermented food to act as a probiotic carrier. The gelatinized potato gave a matrix similar to semisolid dough with firm texture and pale yellow color. Due to this features and because the product was developed through lactic acid fermentation, it was called Potato Cheese. The protocol of processing is described in Fig. 2.

Fermentation was stopped after 6 h to obtain a product with final LAB concentration of ca. 8.0 Log CFU/g and pH 5.1 ± 0.5, which could be optimum for sensory and functional characteristics (Peres et al., 2012).

NaCl (1.5%) and oregano (0.8%) were added to improve organoleptic characteristics and *L.* brevis CJ25 growth was assessed comparing viable LAB counts in CH puree with and without additives (Fig. 3). Although antimicrobial properties of spices against pathogenic or spoilage microorganisms have been documented

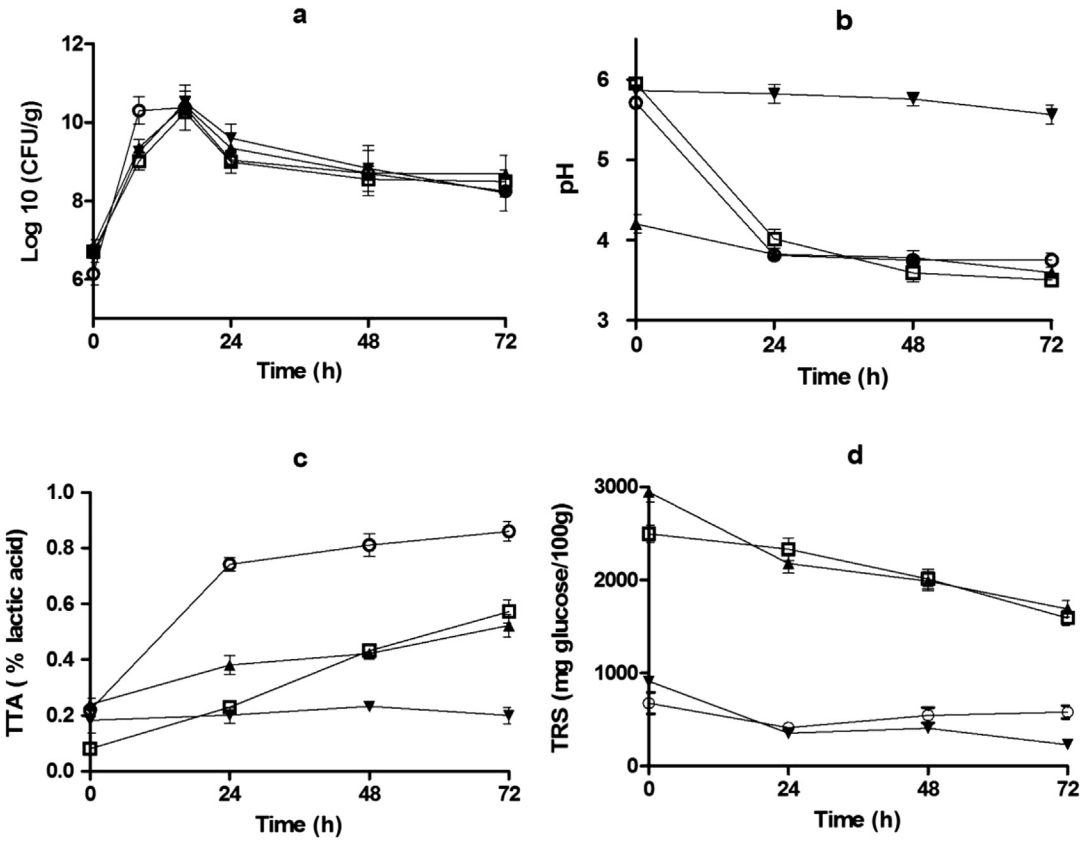

Fig. 1. Changes in pH, viable cell counts, TTA and TRS through 72 h fermentation of SP (▼), OC (□), PL (▲) and CH (○) substrates with *L. brevis* CJ25. The plotted values correspond to means.

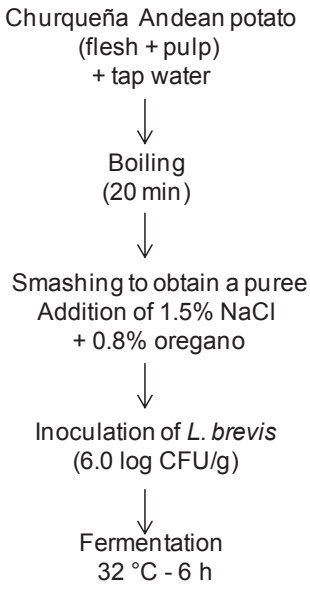

Fig. 2. Biotechnological Protocol for making Potato Cheese.

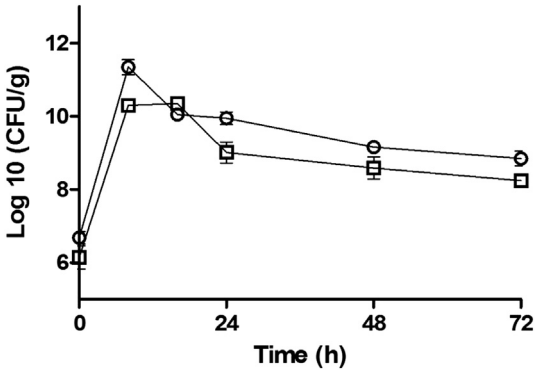

Fig. 3. Viable *L. brevis* CJ25 counts in CH potato (□) and Potato Cheese (○) at 72 h fermentation.

(Gobbetti, Di Cagno, & De Angelis, 2010), only a few reports on LAB cultures are available. Investigations suggest that LAB are relatively resistant to the toxic effect of spices, furthermore, some exert a stimulatory effect on these organisms (Zaika & Kissinger, 1981). Marhamatizadeh et al. (2012) obtained probiotic milk and yoghurt using two LAB strains (*Lactobacillus acidophilus* and *Bifidobacterium bifidus*) and observed that oregano stimulated their growth and acid production. The presence of 0.5–1.0 g/l of oregano increased 2.5 times the TTA and cell counts were higher than control without the spice during 7 days of fermentation, but increasing concentrations progressively delayed bacterial growth and acid production. On the other hand, Kivanc, Akgul, and Dogan (1991) reported that 0.5% of oregano inhibited growth of two strains of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*, although acid production by *L. plantarum* was stimulated. Therefore, inhibitory or stimulatory effects of spices are strain-specific and also depend on their concentration. In this case, Potato Cheese (CH puree + 1.5% NaCl + 0.8% oregano) had slight but significantly ($P < 0.05$) higher values of viable cells than the puree without additives during 72 h of fermentation, indicating that oregano used in the concentration analyzed may stimulate metabolic activities of *L. brevis* CJ25.

The composition of Potato Cheese was: moisture, 66.30 ± 1.86%; proteins (N × 6.25), 8.99 ± 1.82% of dry matter (d.m.); lipids, 0.21 ± 0.03% of d.m.; ash, 4.07 ± 0.65% of d.m.; and carbohydrates, 86.73% of d.m. pH, 5.1; aw, 0.979 and viable LAB content, 8.5 log CFU/g.

3.2. Survival of *L. brevis* CJ25 to cold storage

The effect of cold storage on total count is shown in Fig. 4. Viable cell counts of *L. brevis* CJ25 contained in Potato Cheese were above 8.0 log CFU/g even after 28 days of storage at 4 °C; furthermore, slight cell population increases were found weekly, but not statistically significant ($P < 0.05$). This could be attributed to the presence of residual monosaccharides and disaccharides left after fermentation which provided essential growth nutrients. The same trend was found by Jaiswal and Abu-Ghannam (2013) in cabbage juice fermented with *L. brevis*, *L. plantarum* and *Lactobacillus rhamnosus*, a slight increase (ca. 1.0 log CFU/ml) in the bacterial growth was found after 30 days of storage at 4 °C. Kim et al. (2012) obtained similar results with *Lactobacillus casei* during cold storage of different colored-potatoes beverages, finding that cell counts of fermented "Haryoung" juice remained at 9.0 log CFU/ml during 4 weeks. Notwithstanding, cell concentration decreased from 8.0 to 5.0 log CFU/ml in "Hongyoung" juice, indicating that some food characteristics, such as composition, pH and consistency, could offer protection to LAB during storage, as reported previously by Champagne and Gardner (2008). In the present study, pH decreased 0.7 units during storage period which indicated an active microbial metabolism. A final pH of ca. 4.5 could enhance the stability of LAB and improve food safety avoiding the growth of spoilage and pathogenic microorganisms, such as *Listeria* and *Salmonella*, and represents an alternative to preservatives (Matias, Bedani, Castro, & Saad, 2014). In this way, Enterobacteria were not found in Potato Cheese after 28 days of cold storage and yeasts were detectable after 21 days (20 UFC/g).

3.3. Tolerance to GI simulated conditions

Besides being able to survive in a food matrix throughout shelf-life, probiotic LAB must resist the environmental conditions of the GI tract. In order to assess the tolerance of *L. brevis* CJ25 contained in Potato Cheese to gastric conditions, an *in vitro* methodology was carried out and results are shown in Table 1. After 3 h incubation in PBS at pH 2.5 the number of viable LAB decreased from 8.0 to 6.0 log CFU/g. This result indicated that the strain exhibited high level of survival in simulated gastric juice when delivered in Potato Cheese. This is in agreement with findings for other LAB strains like *L. casei* 373; *L. rhamnosus* GG 53103; Yomix 205 (a probiotic mix containing *L. bulgaricus*, *L. acidophilus*, *Bifidobacterium* spp. and *Streptococcus thermophilus*) (Abadía-García et al., 2013).

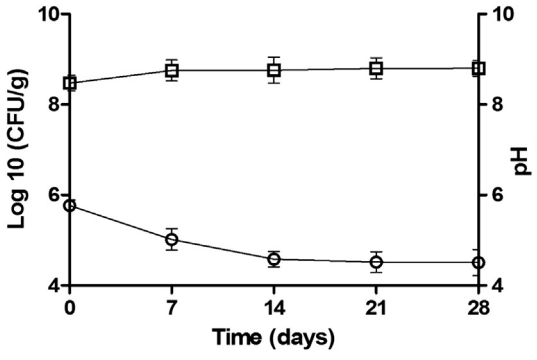

Fig. 4. Viable LAB counts (□) and pH change (○) in Potato Cheese throughout 28 days of storage at 4 °C.

Table 1
Viability Losses during *in vitro* GI treatment.

Culture condition	Decrease of cell counts (log CFU/ml) after treatment		
	Acid (pH 2.5)	Bile (0.3%)	Combined treatment (acid + bile)
L. brevis CJ25, grown in Potato Cheese	2.09 ± 0.13[a]	nd	2.04 ± 0.09[a]
L. brevis CJ25, washed	1.97 ± 0.12[a]	nd	2.02 ± 0.10[a]

Mean ± SD (n = 3). Values with different letter in the same column are significantly different ($P < 0.05$). nd: no detected.

Additionally, *L. brevis* CJ25 contained in Potato Cheese was found to be resistant to bile salts even after 3 h incubation, without reduction in cell counts. After the combined treatment (low pH + bile salt) the viable count decreased around 2.0 log CFU/g from the initial concentration, indicating that only the low pH treatment affected viability.

Some authors (Du Toit et al.,1998; Argyri et al., 2013) suggested that LAB could be protected by food or other carrier matrix molecules following consumption, in this way it was important to assess if the strain had an intrinsic resistance or if the food matrix could affect the stability of the microorganism. The washed strain was exposed to GI conditions and the results did not differ significantly ($P < 0.05$) with those obtained with *L. brevis* contained in Potato Cheese, indicating that the presence of food ingredients had no influence in cell viability.

4. Conclusions

Results showed the suitability of three Andean tubers to be used as raw material for lactic acid fermentation with the potentially probiotic *L. brevis* CJ25 strain. Especially Churqueña potato allows the development of a solid fermented food, called Potato Cheese. Potato Cheese is proposed as a good LAB carrier because the strain remains viable after four weeks of storage at 4 °C, showing metabolic activity with concomitant pH decrease, which prevented contamination by pathogenic and spoilage microorganisms. *L. brevis* CJ25 could also survive the GI tract at significant levels. Due to these desirable *in vitro* probiotic properties, this strain is considered a good candidate for further investigations. As consumer demand for nondairy-based probiotic products has enhanced in recent years, Potato Cheese is a promising novel functional food with high nutritional value, free of cholesterol and lactose and its development may convey a favorable impact in regional economies.

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