

ORIGINAL ARTICLE

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Production of nondairy fermented products with chestnut puree as substrate and milk kefir grains or two lactic acid bacteria

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Abstract

This work aimed to study the production, for the first time, of three fermented products of chestnut puree (CP) with milk kefir grains, a higher nisin-producing (*Lactococcus (L.) lactis* CECT 539) and a higher lactic acid-producing (*Lactobacillus (Lb.) casei* CECT 4043) lactic acid bacteria (LAB). After studying the CP rheology, the ability of the two LAB to assimilate the carbon sources (starch, sucrose, glucose, and fructose) present in the CP was assayed. The two LAB grew well on glucose, fructose, and sucrose, but they did not use starch. Fermentation of CP with the two LAB and kefir grains allowed us to obtain three fermented products that contained no alcohol and low levels of sugars, lactic, and acetic acids, as well as viable cell counts. After 24 h of fermentation, bacterial counts increased by 2.12 log₁₀ cycles for *Lb. casei* and 2.44 log₁₀ cycles for *L. lactis*. Although the final counts for both LAB were very similar ($p > 0.05$), *L. lactis* grew faster than *Lb. casei* and produced a higher concentration of lactic acid. The total microbial counts for kefir increased by 1.16 log₁₀ cycles, after 28 h of incubation, indicating that the microorganisms of the kefir grains adapted well to the composition of the CP. As no ethanol was detected, these results suggest the possibility of producing healthy, nonalcoholic, and low-calorie CPs.

KEYWORDS

chestnut puree, fermented product, kefir grains, kefir-like product, lactic acid bacteria

1 | INTRODUCTION

In recent years, more and more people are concerned about their health and well-being. Consequently, they prefer to consume good quality foods with healthy properties. The consumption of fermented products is in-line with this idea because of their several potential health benefits, especially from those containing lactic acid bacteria (LAB) or yeasts, such as those prepared with kefir grains.

Kefir has been consumed for hundreds of years by Caucasian communities and became very popular during the 20th century. This fermented drink is usually prepared by fermentation of cow's milk with kefir grains, which contains a consortium of yeasts and bacteria (LAB and acetic acid bacteria), being this food widely recognized as an excellent source of microorganisms with potential health effects (Bazán, 2020). However, in addition to lactose intolerance, dairy foods intake has been associated with other negative health effects, including milk protein allergy as well as the increase in the concentrations of total cholesterol in the blood. An appropriate alternative to avoid these health risks could be the use of nondairy products (such as fruit or vegetable juices, desserts, and cereal-based products) as a vehicle for delivering probiotics (Diya et al., 2018).

Chestnut fruits are mainly composed of water and carbohydrates (mostly starch and other sugars such as sucrose and, in less proportion, glucose, fructose, and maltose), proteins with essential amino acids, dietary fiber, minerals, vitamins, and phenolic compounds (gallic and ellagic acids), containing a low amount of fat (Boscaino et al., 2017; De Vasconcelos et al., 2010; Delgado et al., 2010). In addition, chestnut is a naturally gluten-free product, making it an excellent alternative food for celiac people.

The fruits with good caliber and no cracks are destined for sale in fresh, frozen, and gastronomy (marron glacé or candied chestnuts). The low-caliber fruits, polyspermic and broken, are usually discarded and used to produce animal feed, flour, or alcoholic beverages (such as beer) (Fernandes et al., 2020). Considering that Portugal and Spain are among the main producers of chestnut (*Castanea sativa* Miller) in Europe (FAOSTAT, 2021), utilization of this fruit for the production of a fermented nondairy product could be an excellent alternative and economically profitable way to valorize and take advantage of the surpluses of chestnut production. This approach could contribute to promoting the sustainability of chestnut production and respecting the principles of the circular economy (European Parliament, 2023).

In an effort to valorize the fruits with low commercial value to develop an alternative nondairy fermented food, this work studied, for the first time, the fermentation of chestnut puree (CP) with milk kefir grains or two

LAB. First, a rheological study of the CP was conducted to characterize the fermentation substrate. Afterward, the fermentations of CP with *Lactobacillus (Lb.) casei* subsp. *casei* CECT 4043 (a high lactic acid producer) (Bernárdez, et al., 2008a) and *Lactococcus (L.) lactis* subsp. *lactis* CECT 539 (a high nisin producer) (Costas et al., 2018) and kefir grains (Bazán et al., 2022) were conducted as three types of fermentation. Both bacterial strains were selected considering that these two LAB fulfill some probiotic criteria, as indicated by Guerra et al. (2007). This will allow us to offer a wider range of fermented products from chestnut, particularly from fruits with low commercial value. This approach could benefit farmers engaged in chestnut cultivation by increasing their income through the production of a new commercial product, as well as enhancing the value of chestnuts. To characterize and compare the fermentation kinetics of each fermentation entity, different culture variables were measured during the incubation, including the microbial counts, pH, total sugars (TS), reducing sugars, individual sugars, and organic acids.

2 | MATERIALS AND METHODS

2.1 | Solvents and reagents

The Folin–Ciocalteu's reagent, chloramphenicol 98.5%, and sucrose 99% were obtained from Panreac AppliChem. The sodium chloride salt 99.5% and glacial acetic acid were provided by Merck. Phenol 99.5%, absolute ethanol and sodium carbonate 99.8%, and cupric sulfate pentahydrate 99% were supplied by Riedel-de Haen. The hydrazine sulfate 99% and potassium sodium tartrate tetrahydrate 99.5% were obtained from Chemlab. The sulfuric acid 95%–97% and lactic acid 88%–92% were purchased from Fluka. The 3,5-dinitrosalicylic acid (DNS) was provided by Alfa Aesar. D-(+)-glucose anhydrous 97.5%–102.0% was supplied by Scharlau. The sodium hydroxide 98.97% and sodium tartrate dihydrate 99%–101% were obtained from Fischer. The bovine serum albumin 96%, D(-)-fructose 99%, and D-(+)-maltose monohydrate 99% were purchased from Sigma.

For the microbiological analysis, the De Man, Rogosa, and Sharpe (MRS) agar and broth and Rose Bengal chloramphenicol agar (RBCA) were obtained from Liofilchem.

2.2 | Bacterial strains and milk kefir grains origin

The two LAB strains, *Lb. casei* subsp. *casei* CECT 4043 (*Lb. casei*) and *L. lactis* subsp. *lactis* CECT 539 (*L. lactis*) used in this study, were acquired from the Spanish Type

Culture Collection (CECT, Valencia, Spain). Kefir grains were provided from Kefiralia, a trademark of the company Burumart Commerce SL. The commercial product supplied by Kefiralia containing the kefir grains embedded in milk (coverage liquid) was stored at 4°C until further use. The activation of the kefir grains was carried out as indicated by Bazán et al. (2022). For this purpose, the commercial product was filtered using a sterile fine mesh plastic strainer to separate the kefir grains from the coverage liquid. Subsequently, the kefir grains were washed with sterile distilled water and transferred to a sterile glass bottle containing 1 L whole UHT (ultrahigh temperature) milk (Terra Nostra, Azores) in a biosafety cabinet previously sterilized with ultraviolet light for 15 min. The mouth of the bottle was then covered with a sterile cloth (to allow the crop to breathe and prevent the entry of dust), and a rubber band was placed around the cloth and the neck of the bottle (Bazán et al., 2022). The bottle containing the kefir grains and UHT milk was incubated at 20°C in static conditions and protected from light. This procedure was repeated twice by changing the UHT milk every 24 h to maintain the viability of the kefir grains. For longer storage times, such as one week or extended storage, the kefir grains were kept in UHT milk at 4 or –20°C, respectively.

2.3 | Chestnut puree preparation

CP was prepared according to the procedure described by López et al. (2004) and Blaiotta et al. (2012), with some modifications. Raw chestnuts were slightly steamed to remove external teguments and chopped. The broken fruits were mixed with distilled water (1:4, w/v) in a glass pot and homogenized by ultra turrax (IKA, T25 basic) for 2 min at 11,000 rpm. The pH was measured with a pH meter (WTW Inolab, electrode WTW SenTix 41). The suspensions (CPs) were heated in an autoclave (SELECTA, Autester-E 75 Dry) at 121°C for 1 h for simultaneous gelatinization and sterilization. The obtained puree was used as the substrate for rheology determinations and fermentations.

2.3.1 | Chestnut puree rheology

The rheological measurements of the CP were carried out using a rotational, concentric cylinder viscometer (Visco Star plus, Fungilab), coupled to a temperature controller (Electrotemp, Fungilab). A volume of 10 mL of sample was used with the TL6 spindle (0.94 cm diameter) for each determination. The data acquisition was performed by the software “Data Logger vCli 1.01.”

The shear stresses and viscosities were measured at different shear rates (1.7, 3.4, 6.8, 10.2, 17, 34, and 68 s⁻¹) and temperatures (15, 25, 50, and 75°C). The upward and downward curves were also assessed to verify a non-time-dependent flow behavior. All measurements were done in triplicate.

Many fluid foods do not have a Newtonian behavior. Thus, their viscosities can depend on the shear rate or shear stress and vary with temperature. In CP, it was necessary to develop models that could describe the behavior of these fluids. The Ostwald–de Waele (or Power law) model (Equation 1) and Herschel–Bulkley model (Equation 2) are the most widely used rheological models (Maceiras et al., 2007):

$$\tau = K \times \dot{\gamma}^n \quad (1)$$

$$\tau = \tau_{\text{OHB}} + K \times \dot{\gamma}^n \quad (2)$$

where τ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s⁻¹), n is the flow behavior index (dimensionless), K is the consistency coefficient (Pa s ^{n}), and τ_{OHB} is the yield stress (Pa).

The Arrhenius model was applied to determine the temperature dependency of the apparent viscosity at a specific shear rate:

$$\eta = k_0 \times e^{E_a/RT} \quad (3)$$

where η is the apparent viscosity (Pa s), k_0 is a pre-exponential constant (Pa s), E_a is the activation energy (J mol⁻¹), R is the universal gas constant (8.314 J mol⁻¹ K⁻¹), and T is the absolute temperature (K).

2.4 | Fermentations

In this study, three fermentations with CP were conducted. The first, second, and third fermentations were conducted with *Lb. casei*, *L. lactis*, and the previously activated kefir grains, respectively. In the following sections, these fermentations will be named Fermentations I, II, and III, respectively.

2.4.1 | Lactic acid bacteria fermentations

Each lactic acid bacterium (strains *Lb. casei* or *L. lactis*) was preincubated for 10–12 h in MRS broth at 30°C and 100 rpm in a shaking incubator (Shel Lab, SI4-2). The bacterial concentration in this pre-culture was adjusted to an absorbance between 0.8 and 1.0 at 600 nm in a UV–Vis spectrophotometer (JASCO, V-530).

Fermentations were carried out in triplicate glass pots containing CP (30 g chestnut/120 mL water), which was inoculated with 2 mL of each bacterial suspension (strains *Lb. casei* or *L. lactis*) and incubated at 30°C and 200 rpm for 24 h. Sample aliquots were withdrawn aseptically at time intervals during the fermentation process for analytical and microbiological determinations.

2.4.2 | Kefir grains fermentations

First, the kefir grains were washed with sterile distilled water using a sterile fine mesh plastic strainer. Then, the grains were transferred to an Erlenmeyer flask for activation during 24 h in MRS broth (15 g kefir grains/250 mL MRS broth) at 20°C.

Afterward, the CP fermentation was conducted in batch conditions in several 100 mL Erlenmeyer flasks with kefir grains (2 g kefir grains/40 g CP) at 20°C without stirring and protected from light for 28 h. One Erlenmeyer flask containing the fermented puree was collected at set intervals, and its content was homogenized in a Stomacher (VWR, Star Blender LB 400) for 4 min. After homogenization, two aliquots were collected for microbiological and analytical determinations. These fermentations were made in triplicate Erlenmeyer flasks.

2.5 | Microbiological analysis

2.5.1 | Enumeration of bacteria and yeasts

LAB were enumerated by the pour plate method in MRS supplemented with 200 mg cycloheximide/L to inhibit the yeast's growth (Bazán et al., 2022). The yeasts were enumerated by the spread plate method using the RBCA. Chloramphenicol was used to inhibit the growth of bacteria.

For bacteria and yeasts, the plates were incubated at 30°C for 48 h, and 20°C for 5 days, respectively.

2.5.2 | Assimilation of starch, sucrose, glucose, and fructose by the LAB and kefir grains microorganisms

To study the assimilation of starch, sucrose, fructose, and glucose by *Lb. casei* and *L. lactis*, four different culture media with the following composition were prepared: meat peptone (3 g L⁻¹), bromocresol purple (0.025 g L⁻¹), agar (12 g L⁻¹), and the corresponding sugar (10 g L⁻¹). A culture medium without sugars, used as a control, was prepared to study the effect of meat peptone in the growth of these LAB.

Ten microliters of an overnight culture of each lactic acid bacterium in MRS broth were dropped on the prepared agar medium surface and incubated at 30°C for 48 h. The size and color of the halo zone in the agar plate were measured. Each assay was performed in triplicate.

2.6 | Analytical determinations

The pH values of the fermented CPs were measured with a digital pH meter (Hanna, Edge). After that, 1 g of fermented CP was collected and transferred to a sterilized centrifuge tube. Subsequently, 9 mL of sterile distilled water was added, and the mixture vortexed (Heidolph, Reax top) for 30 s. The mixture was centrifuged at 18,031 × g (Eppendorf, 5810R) at 4°C for 15 min. The supernatant was filtered through 0.2 μm nylon filters and stored in sterilized Eppendorf microtubes at -20°C, for further analytic determinations.

2.6.1 | Total sugars

The TS concentration was determined by using the phenol/H₂SO₄ method described by Dubois et al. (1956). Thus, 1 mL of sample, 1 mL of 50 mg mL⁻¹ phenol solution, and 5 mL of 5 mg mL⁻¹ hydrazine sulfate solution were mixed and stirred in a vortex (Heidolph, Reax Top). After cooling at room temperature, the absorbance of the reaction mixture was measured at 490 nm in a UV-Vis spectrophotometer (Jasco, V530). Glucose (at concentrations between 12.5 and 125 mg L⁻¹) was used as standard to build the calibration curve (TS vs. absorbance).

2.6.2 | Reducing sugars

The reducing sugars were determined by the method described by Bernfeld (1955) by mixing 1 mL of the sample with 1 mL of DNS solution and incubating the mixture of reaction for 5 min in a boiling water bath. After cooling, 9 mL of distilled water was added, and the absorbance was measured at 540 nm in a UV-Vis spectrophotometer (Jasco, V530). The calibration curve was prepared using glucose (at concentrations between 0.25 and 2.5 mg mL⁻¹) as standard.

2.6.3 | HPLC analysis

Glucose, maltose, fructose, lactic acid, acetic acid, and ethanol present in the fermented samples were tentatively quantified by using an HPLC system (Agilent 1200 series) equipped with a Rezex ROA-Organic acid H+ column

(Phenomenex). A 0.003 M H₂SO₄ aqueous mobile phase at a flow rate of 0.6 mL min⁻¹ was used to separate sugars and organic acids, and the temperature of the column was 60°C. The different compounds were detected by a refractive index detector at 40°C. The pure standards used to quantify the different sugars and organic acids were D-(+)-glucose, D-(-)-fructose 99%, D-(+)-maltose monohydrate 99%, lactic acid, acetic acid, and absolute ethanol at concentrations between 0.5 and 10 g L⁻¹.

2.7 | Statistical analysis

The normality of the data was verified by the Shapiro–Wilk test, and the homogeneity of the variances was verified by Levene’s test. As the data followed a normal distribution, a one-way independent analysis of variance (ANOVA) with the Tukey (in case of equal variances) or Games–Howell (in case of unequal variances) post hoc tests were used for the comparison of the culture pH and the concentrations of viable cell counts (VCCs), total and reducing sugars, glucose, fructose, maltose, lactic acid, and acetic acid in the unfermented and fermented CP with *Lb. casei*, *L. lactis*, and milk kefir grains. The differences were considered significant for *p*-values <0.05. This statistical analysis was carried out using the software package IBM SPSS Statistics for Windows (Version 21.0, IBM SPSS Inc., 2012) (Bazán et al., 2022).

The values and statistical significance of the constants of the Ostwald–de Waele, Herschel–Bulkley, and Arrhenius models were obtained using the statistics software SigmaPlot for Windows version 12.0 (Systat Software, Inc., San Jose, CA, USA, 2012). The constants of the models were considered statistically significant when their corresponding *p*-values were lower than 0.05.

3 | RESULTS AND DISCUSSION

3.1 | Chestnut puree rheology

The rheological study plays an important role in designing and optimizing the processes, and controlling quality in the food industry. Many factors can affect the rheology of the puree, such as solids content and heat treatment temperatures, among others. Consequently, the rheological data provide information about the structural behavior of food, allowing the correct pump sizing, piping, heat exchangers, stirring operations, and product packaging (Guimarães et al., 2020). In addition, the rheological study may provide information about quality control, sensory analysis, and shelf-life, which are important factors that affect product acceptance (Mathias et al., 2013). The CP to

be used in the fermentations was autoclaved before being inoculated. However, at an industrial level, the puree may simply be pasteurized. It was therefore considered important to analyze its rheological behavior when subjected to different temperatures.

A nonlinear relationship between the apparent viscosity and shear rate ($\dot{\gamma}$) was observed, indicating a non-Newtonian behavior of the CPs (Figure S1). In fact, the apparent viscosity of CP samples decreased as the applied shear rate increased, showing a shear-thinning behavior. Furthermore, all samples demonstrated thixotropic characteristics, observed by the difference between the upward and downward curves, known as hysteresis (Mathias et al., 2013). Hysteresis can occur due to the breakdown of the gel and can be quantified as the area between the upward and downward flow curves. A larger area between the curves indicates a more significant thixotropic effect. Furthermore, the upward and downward curves presented a more substantial hysteresis for the lower temperatures (Figure S1). So, as the temperature increased, the hysteresis became more negligible. This fact agrees with the results of Witczak et al. (2011), who also observed that increasing temperature reduces thixotropy.

The non-Newtonian behavior was also stated when representing the shear stresses as a function of shear rate at 15, 25, 50, and 75°C. Satisfactory results were obtained when the Herschel–Bulkley and Ostwald–de Waele models were fitted to the experimental data (Figure 1). As can be noted, both models gave similar results and fitted well with the experimental data, providing high determination coefficients (0.9712–1.0000, respectively).

The parameters determined for the Ostwald–de Waele and Herschel–Bulkley models are indicated in Table 1.

The yield shear stress, incorporated in the Herschel–Bulkley model, is a parameter that indicates when the material behavior changes from elastic to viscous and quantifies the amount of stress that the fluid may experience before it begins to flow. The yield shear stress varied between negative values and 5.71 Pa, showing that this parameter is insignificant. Thus, the Ostwald–de Waele model is more adequate than the Herschel–Bulkley model to fit the experimental results of CP. Moreover, for both models, it was stated that the flow behavior index (*n*) was lower than one, confirming the classification of the puree as a non-Newtonian fluid with shear-thinning characteristics.

As observed in Figure 1, there was a decrease in the shear stress as the temperature increased. Furthermore, when the temperature increased, an increase in the flow index (lower shear-thinning) was noted. On the contrary, the consistency coefficient decreased, varying from 36.68 to 6.34 Pa s^{*n*} at 15 and 75°C, respectively (Table 1).

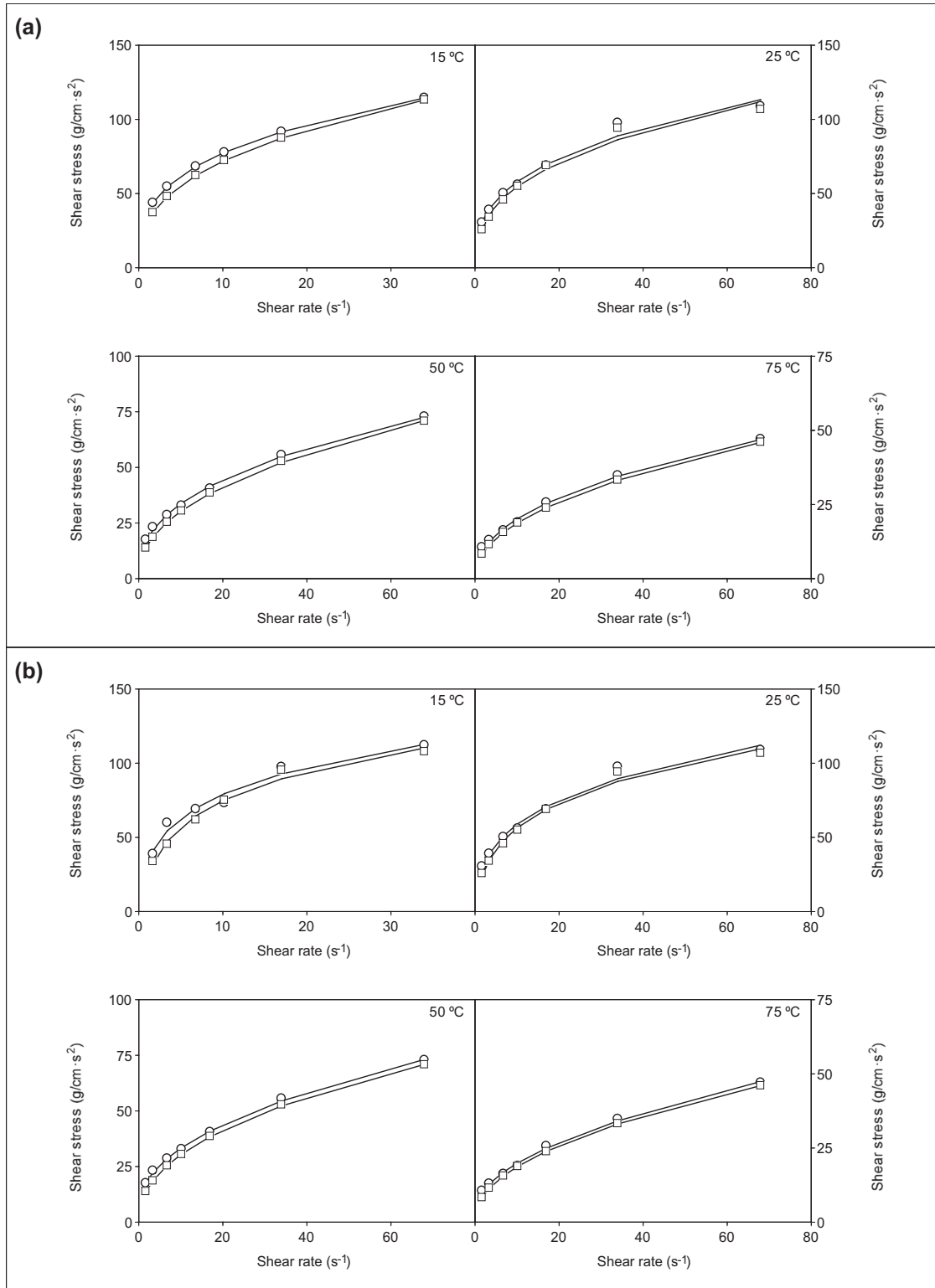


FIGURE 1 Downward and upward curves for the dependence of the shear stress on the shear rate of chestnut puree at four temperatures. The lines drawn through the experimental data (open circles and squares) were obtained according to the Ostwald–de Waele model (a) or the Herschel–Bulkley model (b).

TABLE 1 Statistical significant ($p < 0.05$) rheological parameters (as coefficient value \pm standard error) of the Ostwald–de Waele and Herschel–Bulkley models for chestnut puree at different temperatures.

Ostwald-de Waele model								
<i>T</i>	<i>Upward curve</i>			<i>Downward curve</i>				
	<i>K</i>	<i>n</i>	<i>R</i> ²	<i>K</i>	<i>n</i>	<i>R</i> ²		
15	36.68 \pm 3.25	0.32 \pm 0.03	0.9672	30.43 \pm 3.05	0.37 \pm 0.03	0.9712		
25	25.79 \pm 2.15	0.35 \pm 0.02	0.9799	22.94 \pm 2.09	0.38 \pm 0.03	0.9805		
50	13.39 \pm 0.45	0.40 \pm 0.01	0.9976	10.85 \pm 0.12	0.45 \pm 0.00	0.9998		
75	7.27 \pm 0.38	0.44 \pm 0.01	0.9956	6.34 \pm 0.06	0.47 \pm 0.00	0.9999		
Herschel-Bulkley model								
<i>T</i>	<i>Upward curve</i>				<i>Downward curve</i>			
	τ_{OHB}	<i>K</i>	<i>n</i>	<i>R</i> ²	τ_{OHB}	<i>K</i>	<i>n</i>	<i>R</i> ²
15	-69.83 \pm 1.79	101.4 \pm 3.74	0.17 \pm 0.01	0.9721	-145.68 \pm 1.37	166.29 \pm 3.02	0.12 \pm 0.01	0.9863
25	-19.64 \pm 1.49	42.03 \pm 2.36	0.27 \pm 0.02	0.9820	-45.27 \pm 1.22	61.15 \pm 2.12	0.22 \pm 0.01	0.9885
50	5.71 \pm 0.25	9.37 \pm 0.27	0.47 \pm 0.01	0.9988	-1.12 \pm 0.09	11.62 \pm 0.11	0.43 \pm 0.00	0.9998
75	3.77 \pm 0.25	4.81 \pm 0.25	0.52 \pm 0.01	0.9974	0.76 \pm 0.03	5.84 \pm 0.03	0.49 \pm 0.00	1.0000

Note: *T* is the temperature ($^{\circ}$ C), *K* is the consistency index (Pa s^{*n*}), *n* is the flow behavior index (dimensionless), *R*² is the determination coefficient, and τ_{OHB} is the yield stress (Pa).

TABLE 2 Statistically significant ($p < 0.05$) pre-exponent constant (*K*₀, in Pa s) and activation energy (*E*_a, in kJ mol⁻¹) values (as coefficient value \pm standard error) of the Arrhenius model applied to determine the temperature dependency of the apparent viscosity at a specific shear rate (s⁻¹) for the upward and downward curves.

Shear rate	Upward curve			Downward curve		
	<i>K</i> ₀	<i>E</i> _a	<i>R</i> ²	<i>K</i> ₀	<i>E</i> _a	<i>R</i> ²
1.7	1.26 $\times 10^{-3} \pm 9.62 \times 10^{-5}$	17.95 ± 0.19	0.9999	5.36 $\times 10^{-4} \pm 2.48 \times 10^{-5}$	19.67 ± 0.11	1.0000
3.4	0.20 $\times 10^{-4} \pm 0.21 \times 10^{-5}$	21.71 ± 2.60	0.9836	3.85 $\times 10^{-4} \pm 6.42 \times 10^{-5}$	19.50 ± 0.41	0.9995
6.8	2.46 $\times 10^{-4} \pm 8.13 \times 10^{-5}$	19.91 ± 0.81	0.9980	2.60 $\times 10^{-4} \pm 5.85 \times 10^{-5}$	19.51 ± 0.55	0.9990
10.2	3.47 $\times 10^{-4} \pm 6.78 \times 10^{-5}$	18.27 ± 0.48	0.9991	1.92 $\times 10^{-4} \pm 5.81 \times 10^{-5}$	19.73 ± 0.74	0.9983
17.0	1.86 $\times 10^{-4} \pm 1.13 \times 10^{-5}$	19.18 ± 1.50	0.9923	1.41 $\times 10^{-4} \pm 5.50 \times 10^{-5}$	19.82 ± 0.96	0.9971
34.0	4.45 $\times 10^{-4} \pm 2.61 \times 10^{-5}$	15.89 ± 1.45	0.9890	3.98 $\times 10^{-4} \pm 2.47 \times 10^{-4}$	16.06 ± 1.53	0.9880
68.0	5.73 $\times 10^{-4} \pm 2.11 \times 10^{-5}$	13.97 ± 0.95	0.9961	5.33 $\times 10^{-4} \pm 1.68 \times 10^{-4}$	14.09 ± 0.81	0.9972
Mean	4.66 $\times 10^{-4}$	18.13		3.49 $\times 10^{-4}$	18.34	
SD	3.80 $\times 10^{-4}$	2.56		1.58 $\times 10^{-4}$	2.30	
<i>C</i> _V	81.62	14.14		45.10	12.56	

Note: *C*_V is the coefficient of variation (%), *SD* is the standard deviation, and *R*² is the determination coefficient.

Concerning CP heating, for all cases, it was found that the viscosity decreased with temperature (Figure S2). When the Arrhenius model was applied to determine the temperature dependency of the apparent viscosity at a specific shear rate, high determination coefficients (between 0.9836 and 1.0000) were obtained (Table 2). The activation energy and pre-exponent constants are shown in Table 2.

The activation energy reflects the fluid's apparent viscosity dependence on temperature (Witczak et al., 2011). As observed in Table 2, the activation energy remained almost constant at a value of about 18 kJ mol⁻¹ in the temperature range from 15 to 75 $^{\circ}$ C for both upward and downward curves. Thus, these results indicated that the sensitivity of the viscosity values of CP to temperature changes was not significant for the shear rates applied.

3.2 | Starch, sucrose, glucose, and fructose utilization by LAB strains

This experiment was carried out in plates containing four different carbon sources (starch, sucrose, glucose, and fructose). A change in the color of the halo zone, from purple to yellow, is caused by the decrease in pH caused by the production of organic acids by the growing bacterium due to the fermentation of sugars to support the bacterial growth.

L. lactis used fructose, glucose, and sucrose as the preferred carbon source for growth; meanwhile, *Lb. casei* preferentially consumed fructose and glucose as the carbon source, although it also consumed sucrose, but to a lesser extent (Table S1).

The halos of growth measured for *L. lactis* were similar in the media supplemented or non-supplemented with starch, suggesting that this strain did not use the polysaccharide as a carbon source. So that, the halo of growth observed in these two media was probably due to the presence of meat peptone. Regarding the *Lb. casei*, it can be observed that this lactic acid bacterium did not use starch or meat peptone (Table S1).

These results indicated that fructose, glucose, and sucrose were good carbon sources for the growth of *L. lactis*. In the same way, fructose and glucose were also good carbon sources for the growth of *Lb. casei*. In contrast, starch was not a good carbon source for the growth of both LAB. Consequently, strains *Lb. casei* and *L. lactis* cannot be considered amylolytic bacterial strains. That means that these LAB do not produce amylolytic enzymes to hydrolyze starch.

In a previous work, Costas et al. (2018) observed that *L. lactis* CECT 539 could not use the glycogen, a multi-branched polysaccharide of glucose present in a culture medium prepared with mussel processing waste (MPW), and concluded that this strain is not an amylolytic bacterium.

Pintado et al. (1999) screened four LAB (*Lb. plantarum* LMG 18053, *Lb. manihotivorans* LMG 18010T, *Lb. plantarum* R10101/2, and *Pediococcus* sp. VA403) for their amylolytic capability in MPW (containing glycogen), MRS-starch, and MRS-glycogen media. These researchers observed that only *Lb. plantarum* R10101/2 was unable to completely degrade starch or glycogen present in the culture media. These results suggest that the ability to produce amylolytic enzymes is strain-dependent.

3.3 | Chestnut puree fermentation

3.3.1 | Growth of microorganisms on chestnut puree

Figure 2 shows the changes in the total microbial counts during the fermentation of CP with two LAB (strains *Lb. casei* or *L. lactis*), and kefir grains (Fermentations I, II, and III, respectively). As observed, the two LAB exhibited a logistic growth; meanwhile, the microorganisms of the kefir grains grew almost linearly during the complete incubation in CP (Figure 2a).

The initial counts of strains *Lb. casei* and *L. lactis* were 6.89 and $6.58 \log_{10}$ CFU mL⁻¹, respectively. After 24 h of fermentation, bacterial counts increased by $2.12 \log_{10}$ cycles for *Lb. casei* (6.89 ± 0.01 to $9.01 \pm 0.02 \log_{10}$ CFU mL⁻¹) and $2.44 \log_{10}$ cycles for *L. lactis* (6.58 ± 0.09 to $9.02 \pm 0.02 \log_{10}$ CFU mL⁻¹). Although the final counts for both LAB were very similar ($p > 0.05$), *Lb. casei* grew more slowly than strain *L. lactis*.

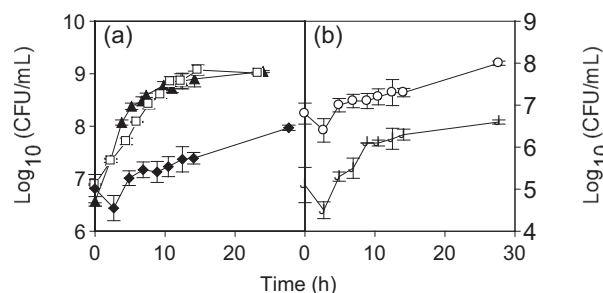


FIGURE 2 (a) Counts of viable cells of *Lactobacillus (Lb.) casei* CECT 4043 (open squares) (Fermentation I), *Lactococcus (L.) lactis* CECT 539 (closed triangles) (Fermentation II), and kefir microorganisms (closed diamonds), as the sum of bacterial and yeast counts (Fermentation III). (b) Independent counts of viable cells of lactic acid bacteria (open circles) and yeasts (J) of the kefir grains during the Fermentation III of the chestnut puree.

For the fermentation with kefir grains, the initial total counts (the sum of bacterial and yeast counts) were $6.81 \pm 0.48 \log_{10}$ CFU mL⁻¹, indicating that part of the microflora contained in the kefir grains was transferred to the CP. The total microbial counts for kefir increased by $1.16 \log_{10}$ cycles (6.81 ± 0.48 to $7.97 \pm 0.07 \log_{10}$ CFU mL⁻¹), after 28 h of incubation (Figure 2a). However, a decrease in microbial counts was observed after 3 h of the inoculation of CP with kefir grains. According to Norberto et al. (2018), the fermentation parameters, such as pH, type of substrate, and concentration of starter culture, may affect the regulation mechanism and the lag time of the microorganisms, producing stress for microorganisms and causing competition and new ways of adaptation. Thus, the different compositions of the fermentation substrate (CP) and the medium (UHT milk) used in the activation of the kefir grains, probably stressed the microorganisms of the kefir grains leading to a reduction in the microbial counts. The logistic increase in the microbial counts from 3 h to the end of incubation (Figure 2a) indicated that the microorganisms of the kefir grains adapted well to the composition of the CP.

The results obtained suggested that the bacterial strains *Lb. casei* and *L. lactis*, as well as the microorganisms of the kefir grains, can use the carbon sources of the CP for growth.

Regarding the enumeration of bacteria and yeasts over time for the kefir grains fermentation (Figure 2b), it can be noted that during 28 h of fermentation, the bacterial concentration increased $1.20 \log_{10}$ cycles (6.75 ± 0.25 to $7.95 \pm 0.05 \log_{10}$ CFU mL⁻¹) and the yeast concentration increased $1.42 \log_{10}$ cycles (5.18 ± 0.42 to $6.60 \pm 0.05 \log_{10}$ CFU mL⁻¹). As observed in Figure 2b, the bacterial concentration was always higher than the concentration of yeasts ($p < 0.05$), probably because the initial concentration of the latter microorganisms ($5.18 \pm 0.42 \log_{10}$

CFU mL⁻¹) was lower than the initial LAB concentration ($6.75 \pm 0.25 \log_{10}$ CFU mL⁻¹).

Liu and Lin (2000), Koh et al. (2017), Magalhães et al. (2010), Norberto et al. (2018), Sabokbar and Khodaiyan (2015), and Silva et al. (2018) also obtained higher VCC of LAB than yeasts in kefir-like beverages obtained by fermentation of different substrates with kefir grains of different origins. However, the increase in the viable yeast counts (as the difference between the final and initial values) in all kefir-like beverages was higher than those of the LAB (Magalhães et al., 2010; Norberto et al., 2018; Silva et al., 2018).

A different trend was observed in the fermentations of deproteinized whey (Agregán-Pérez et al., 2021) or red table grape juices (at initial pH values of 3.99 and 5.99) (Bazán et al., 2022) with milk kefir grains CIDCA AGK1. In these fermentations, the final VCC of yeasts and their increase were higher than those of LAB.

These results indicated that the final VCC of LAB and yeasts and their increase in the kefir-like beverages depend on the fermentation substrate and the kefir grains' origin. In any case, LAB and yeasts coexist in a symbiotic association, and the production of lactic acid by LAB produces acidification of the fermentation substrate that stimulates the growth of the yeasts, which produces growth-stimulating factors (vitamins and nitrogen compounds) that favor the growth of LAB (Magalhães et al., 2010). Both microbial groups are responsible for acid-alcoholic fermentation during the production of kefir-like beverages, being the LAB and yeasts important because of the production of lactic acid (LAB), ethanol, and carbon dioxide (yeasts) (Liu & Lin, 2000).

These results are promising as the VCCs of the three fermented CPs were higher than the minimal dose ($6.0 \log_{10}$ CFU mL⁻¹) recommended for a probiotic product at the time of consumption. In addition, the two LAB and the bacteria and yeasts present in the kefir grains fulfill many of the probiotic properties as these microorganisms: (i) are nonpathogenics, (ii) can be produced at high concentrations, (iii) can produce antibacterial compounds (bacteriocins and organic acids), (iv) are tolerant to the gastrointestinal conditions, (v) can survive during storage with skim milk at -20°C for several months, (vi) able to survive in piglet feed at room temperature for many days, and (v) produce effective effects in the host entity, mainly piglets (Guerra et al., 2007) and broiler chickens (Bernárdez et al., 2008b; Fajardo et al., 2012). According to Bernárdez et al. (2008a), *Lactobacillus* strains are considered to be extremely aciduric bacteria, which can increase their survival in acidic environments due to the production of increased levels of long-chained, mono-unsaturated membrane fatty acids. However, the probiotic properties of the fermented CPs must be determined in the future.

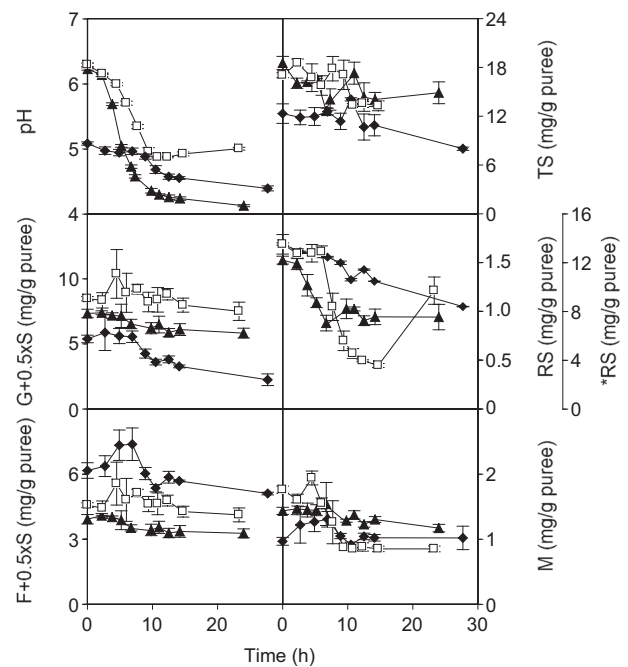


FIGURE 3 Changes in the culture pH, and concentrations of total sugars (TS), reducing sugars (RS), glucose + sucrose (G + 0.5 × S), fructose + sucrose (F + 0.5 × S), and maltose (M) during the fermentation of the chestnut puree by *Lactobacillus* (*Lb.*) *casei* CECT 4043 (open squares) (Fermentation I), *Lactococcus* (*L.*) *lactis* CECT 539 (closed triangles) (Fermentation II), and milk kefir microorganisms (closed diamonds) (Fermentation III). RS: reducing sugars for fermentation with milk kefir grains (Fermentation III).

3.3.2 | Physicochemical parameters during the chestnut puree fermentation

The pH decreased in all fermentations under study, as can be observed in Figure 3. The highest decrease was observed for the fermentation with *L. lactis*, from an initial value of 6.23 ± 0.02 to 4.13 ± 0.02 , after 24 h of incubation. For *Lb. casei*, the lowest pH value was achieved after approximately 11 h of fermentation (4.87 ± 0.01), and its value increased slightly to 5.00 ± 0.02 at the end of the incubation. These results agree with those of Blaiotta et al. (2012), who reported a final pH of about 4.60 for the fermentation of CP (initial pH of about 6.00) with four strains of *Lb. rhamnosus* and two strains of *Lb. casei*.

For the fermentation of CP with kefir grains (Fermentation III), the pH decreased from 5.08 ± 0.03 to 4.39 ± 0.04 . As observed in Figure 3, the initial pH of the fermentation medium after inoculation with the kefir grains was lower than those of the fermentations with *L. lactis* and *Lb. casei*, probably due to the presence of organic acids in the kefir grains. Our results are similar to Norberto et al. (2018), when studying the fermentation of water-soluble soybean (initial pH of 6.50) with kefir grains, which also obtained a final pH value close to 4.00. According to

Sabokbar and Khodaiyan (2015), the reduction in pH of the fermentation substrate after kefir grains inoculation is associated with the production of lactic and acetic acids that inhibit the growth of spoilage and pathogenic bacteria in the fermented beverages.

In the fermentations with the two LAB (Fermentations I and II), the TS consumption was very similar: 4.2 mg g⁻¹ puree (from 18.9 to 14.7 mg g⁻¹ puree) in the case of *Lb. casei*, and 4.1 mg g⁻¹ puree (from 20.6 to 16.5 mg g⁻¹ puree) in the case of *L. lactis*. However, in the fermentation of CP with kefir grains (Fermentation III), the TS consumption was 4.9 mg g⁻¹ puree (from 13.7 to 8.9 mg g⁻¹ puree). These differences in the TS consumption between the two LAB and kefir grains seem to be associated with the higher number of microorganisms in the kefir grains (a consortium of LAB and yeasts) compared to the pure culture of *Lb. casei* or *L. lactis*.

In the CP fermentation with *L. lactis* (Fermentation II), the concentration of reducing sugars (RS) decreased by 38.2%, in the first 7 h of incubation (Figure 3). After that time, a slight increase in the RS concentration was observed, probably due to the production of glucose and fructose due to the hydrolysis of sucrose catalyzed by the enzymes produced by *L. lactis* at the beginning of the stationary phase. From 12.5 to 24 h, the RS concentration remained almost constant. The enzyme-catalyzed hydrolysis of starch would not be considered a cause for the observed increase in RS concentration because *L. lactis* is not an amyolytic strain (Table S1).

On the other hand, in the fermentation with *Lb. casei* (Fermentation I), the RS concentration remained almost constant in the first 6 h (approximately 1.6 mg g⁻¹ puree), decreased abruptly (by 73.5%) to 0.45 mg g⁻¹ puree until the 15 h of fermentation, and increased to 1.2 mg g⁻¹ puree at the end of the incubation. Considering that the VCC of *Lb. casei* (Figure 2a) remained constant from 15 h until the end of the incubation, it could be considered that when the *Lb. casei* cells entered the stationary phase, the rate of sucrose hydrolysis was higher than the consumption rate, increasing the RS concentration in the fermentation medium. Blaiotta et al. (2012) also observed an increase in the concentrations of glucose and fructose during fermentation of CP with *Lb. rhamnosus* RBT739 (during the first 7 h of incubation) and *Lb. casei* strains Lbc491 (during the first 7 h of incubation) and Lbc496 (from the 7 to 24 h of incubation). As observed for *Lb. casei* (Figure 3), Blaiotta et al. (2013) noted a slight consumption of reducing sugars (glucose and fructose) by *Lb. rhamnosus* strains VT1, RBM526, and RBT739 and *Lb. casei* strains Lbc491 and Lbc496 during the first 7 h of fermentation.

The RS for the fermentation with kefir grains (Fermentation III) decreased by 37.9% (from 13.5 to 8.4 mg g⁻¹ puree). The high initial RS concentration in the CP medium inoc-

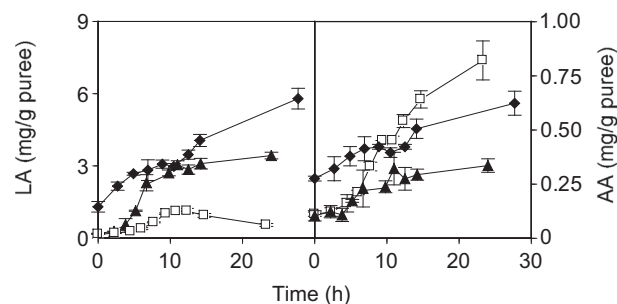


FIGURE 4 Changes in the concentrations of lactic acid (LA) and acetic acid (AA) during the fermentation of the chestnut puree by *Lactobacillus (Lb.) casei* CECT 4043 (open squares) (Fermentation I), *Lactococcus (L.) lactis* CECT 539 (closed triangles) (Fermentation II), and milk kefir microorganisms (closed diamonds) (Fermentation III).

ulated with kefir grains compared with the CP inoculated with *Lb. casei* or *L. lactis* (Fermentations I and II) is due to the kefir grains contain a high level of RS, whereas the raw CP has only around 1.5 mg g⁻¹ puree.

As all sugars (glucose, fructose, maltose, and sucrose) were determined by HPLC, the presence of H₂SO₄ as a mobile phase could cause an acid-catalyzed hydrolysis of sucrose (Bower et al., 2008) producing glucose and fructose. So, in the results, the sum of glucose + 0.5 × sucrose and fructose + 0.5 × sucrose contents was considered.

Regarding the time course of maltose, a slight decrease in the concentration of this disaccharide was observed during the fermentation with strain *L. lactis* and kefir grains (Fermentations II and III, respectively) (Figure 3). Furthermore, this decrease was more pronounced for *Lb. casei* (from 1.91 ± 0.28 to 0.87 ± 0.86 mg maltose g⁻¹ puree) (Fermentation I).

The time course of the production of organic acids (lactic and acetic acids) during the fermentation process is shown in Figure 4. As observed, *Lb. casei* and *L. lactis* (Fermentations I and II) produced lactic acid as the main organic acid and a small amount of acetic acid during the CP fermentation.

Lactic acid increased significantly ($p < 0.05$) from an initial value of 1.30 ± 0.22–5.79 ± 0.43 mg g⁻¹ puree in the CP medium fermented with kefir grains (Fermentation III).

The *Lb. casei* and *L. lactis* fermentations (Fermentations I and II, respectively) were started in CP medium containing a similar initial concentration of lactic acid, 0.16 ± 0.01 and 0.19 ± 0.01 mg g⁻¹ puree, respectively. However, at the end of the fermentation, *L. lactis* produced a higher concentration of lactic acid (3.43 ± 0.14 mg g⁻¹ puree) than *Lb. casei* (0.54 ± 0.06 mg g⁻¹ puree). In the fermentation with the latter strain, the concentration of lactic acid slightly decreased from 10.8 h until the end of fermentation (Figure 4), which parallels with the slight increase

in pH value observed in the *Lb. casei* culture in the same period of time (Figure 3). This slight decrease in the lactic acid concentration was probably due to the assimilation of this compound by the growing strain. This hypothesis is supported by the fact that some *Lactobacillus* strains (*Lb. curvatus*, *Lb. sake*, *Lb. casei*, and *Lb. plantarum*) can metabolize the lactic acid to produce acetate and CO₂ under aerobic conditions (Liu, 2003) and under conditions of carbon source limitation (Pintado et al., 2005). In addition, *Lb. buchneri* LMG 6892^T and *Lb. parabuchneri* LMG 11457^T catabolized lactic acid to produce 0.48 mol of acetic acid, 0.48 mol of 1,2-propanediol, 0.52 mol of CO₂, and traces of ethanol per mol of lactic acid consumed (Elferink et al., 2001). The mechanism of lactate assimilation by LAB is produced by the activities of the enzymes lactate dehydrogenase and pyruvate oxidase and includes the utilization of oxygen as an electron acceptor to form acetate, CO₂, H₂O₂, and an additional ATP (Liu, 2003; Pintado et al., 2005). Probably due to the low concentration of RS (between 1.69 at $t = 0$ h, to 1.21 mg g⁻¹ puree at the end of incubation) in the CP medium (Figure 3), *Lb. casei* (Fermentation I) developed heterolactic fermentation (Figure 4) producing similar amounts of lactic (maximum amount: 1.12 mg g⁻¹ puree) and acetic acid (maximum amount: 0.82 mg g⁻¹ puree).

In contrast, both the milk kefir grains and *L. lactis* produced concentrations of lactic acid (5.79 and 3.43 mg g⁻¹ puree, respectively) 10 times higher than those of acetic acid (0.56 and 0.34 mg g⁻¹ puree, respectively). The production of acetic acid by *L. lactis* could also be attributed to the low levels of reducing sugars in the CP medium (Costas et al., 2018), as described above for *Lb. casei*. According to Magalhães et al. (2010), the production of acetic acid in the milk kefir grains culture could be attributed to the acetic acid bacteria and heterofermentative LAB present in kefir grains microflora. Regarding ethanol, it was not detected in any of the three fermentations. This is particularly surprising in the fermentation using kefir grains, which contain yeasts responsible for ethanol production (Bazán et al., 2022, 2024; Fiorda et al., 2016; Magalhães et al., 2010; 2011). However, ethanol concentrations in kefir beverages can vary significantly depending on fermentation conditions, such as the type, composition, and origin of the substrate, as well as temperature, fermentation time, and the type of starter culture used (Fiorda et al., 2016; Magalhães et al., 2010; 2011). For instance, relatively low ethanol concentrations of 0.04 g L⁻¹ (Fontán et al., 2006), 0.5 g L⁻¹ (Magalhães et al., 2011), 0.02 g L⁻¹ (Gamba et al., 2020), and 3.34 g L⁻¹ (Bazán et al., 2024) have been reported in kefir beverages produced from different milks after 24 h of fermentation. These milk substrates have initial concentrations of sugars (~46 g L⁻¹) considerably higher than that (15.97 ± 3.24 mg g⁻¹) of the CP used in this study. Thus,

the considerably low concentrations of glucose, fructose, and maltose in the fermentation medium during incubation (Figure 3) likely limited the production of ethanol by yeasts.

Additionally, it is well known that species of acetic acid bacteria, such as *Acetobacter* and *Gluconobacter*, can oxidize ethanol to produce acetic acid (Gomes et al., 2018). This suggests that the acetic acid bacteria in kefir grains could assimilate the ethanol synthesized by the yeasts present in the grains, as noted by Magalhães et al. (2011).

Therefore, it seems reasonable to assume that the final concentration of the different metabolic products in kefir-like beverages results from the balance between their production, consumption, or conversion into other compounds by the microbial population of the kefir grains (Bazán et al., 2024). These hypotheses may provide a possible explanation for the absence of ethanol in the kefir-like chestnut product.

The large microbial diversity prevailing in kefir grains and its easy adaptation to different substrates are advantages when compared to single-species starter cultures (Norberto et al., 2018).

4 | CONCLUSIONS

This study provides a novel and efficient approach to produce fermented CPs at low cost obtained by fermentation of fruits with a low commercial value with one LAB strains (*L. lactis* or *Lb. casei*) or milk kefir grains.

The three fermented products prepared in the present work contained levels of viable cells (9.0 or 8.0 log₁₀ CFU mL⁻¹ in the case of those obtained with the LAB or milk kefir grains, respectively), which suggests that in the future it may be possible to develop a probiotic drink. In addition, the three fermented products are characterized by low levels of organic acids and sugars and did not contain alcohol. Therefore, the production of these products could be a suitable alternative to offer a healthy, new non-alcoholic, low-acid product to consumers, mainly vegans, vegetarians, celiac, and lactose-intolerant people. Thus, the valorization of small and broken chestnut fruits could contribute to reducing the fruit loss and increasing the farmer's income. However, to our knowledge, this is the first study that proposed the CP as a nondairy substrate for the fermentation with milk kefir grains.

AUTHOR CONTRIBUTIONS

Maria João Afonso: Writing—original draft; investigation; methodology; formal analysis. **Elsa Ramalhosa:** Conceptualization; supervision; validation; funding acquisition; formal analysis. **Pablo G. del Río:** Methodology; funding acquisition. **Fátima Martins:**

Methodology. **Paula Baptista**: Resources. **Ermelinda L. Pereira**: Resources. **Nelson P. Guerra**: Conceptualization; supervision; validation; formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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