

Optimization of Mixing Conditions for improving *Lactobacillus paracasei* CBA L74' s Growth during Lactic Fermentation of Cooked Navy Beans and Functional Characterization of the Fermented Products

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Lactic acid fermentation of legume-based matrices has been gaining an increasing interest in recent years to produce pro- and post-biotic functional foods at high protein content. The optimization of the main process conditions is necessary to improve bacterial growth, microbial metabolic activity, thus the consequent functional properties of the resulting fermented products. A preliminary study of feasibility about the potential of using a leguminous substrate as growth medium for *Lactobacillus paracasei* CBA L74 was already conducted in previous experimentations. In this work, the effect of improving mixing conditions of a cooked navy bean suspension during the lactic acid fermentation process was investigated and some chemical and physical properties of the resulting fermented products were studied to verify their potential application as functional ingredients in food formulations. The mixing system was optimized by designing an impeller that guarantees a more homogeneous distribution of nutrients and avoids concentration gradients that could inhibit the microorganism proliferation. Bacterial growth, lactic acid production, sugar, and starch consumption were compared with those obtained during previous experimentations carried out with a different impeller. The major availability of nutrients in the fermenting medium reached with the customized impeller allowed a faster achievement of the maximum microbial load (1×10^9 CFU/mL after 14 h of process) and a lower doubling time of 1.53 h. Furthermore, fermentation led to a reduction in water absorption, oil binding, and foam capacity of the fermented bean powders. Total phenolic and flavonoid content and their antioxidant capacity were not influenced by the fermentation process itself but favored by the previous thermal treatment of sterilization.

1. Introduction

In addition to dairy based functional foods, considered the most common probiotic substrates for several years, non-dairy based foods have been gaining increasing interest for their gut health enhancing effects and their ability to satisfy a wider range of consumers, even those affected by intolerances, such as lactose and milk protein allergies, high blood cholesterol concentration (Granato et al., 2010), celiac disease, and with different dietary habits. Lactic acid fermentation of cereals, legumes, fruits, and vegetables-based foods can be considered a simple and economical way of improving their nutritional and functional qualities, making them healthy non-dairy carriers to prepare pre, pro, and postbiotic foods. Legumes provide high protein meat-substitutes for vegetarians, low fat substitutes for health-conscious individuals, and low-cost products for low-income groups (Maphosa and Jideani, 2017). Fermentation of leguminous substrates can influence the presence of various bioactive components, such as vitamins, natural phenolics, and bioactive peptides (Gan et al., 2017) and can promote the reduction of anti-nutritional factors, as oligosaccharides, proteinase

inhibitors, and trypsin inhibitors, saponins, and phytates (Worku and Sahu, 2017). Phytochemicals with antioxidant properties, as polyphenols compounds, play a key role in the prevention of some cancers, heart diseases, osteoporosis, and other chronic degenerative diseases (Bouchena and Lamri-Senhadj, 2013). They are mainly found in conjugated form with organic acids or sugar groups, or can be covalently bound to cell wall macromolecules, as polysaccharides and proteins. This can reduce their bioavailability and compromise their health benefits (Rani et al., 2018). Their content can be significantly enhanced by the enzymatic activity of microorganisms (e.g., amylases, xylanases, and glucosidases) and a subsequent release of phenolic and bioactive compounds in free form can occur, as reported by Espinosa-P´aez et al. (2017). Furthermore, fermentation can impact on some properties with technological functionality such as water and oil holding capacity, emulsifying, and foaming properties, which have a fundamental role in processing and formulating food products. Low water absorption is desirable for making thinner gruels, while high values are desirable for the formulation of baked products, which require high hydration to improve the handling properties of doughs (Alka et al., 2012). Oil binding capacity represents an important parameter owing to its influence on industrial processing, shelf-life, and sensory quality of food, such as mouthfeel and flavor retention (Bessada et al., 2019). Foam capacity is dependent on the presence of soluble protein molecules which favor the suspension of air bubbles and decrease their surface tension. Its increase is often combined with increasing water retention capacity and is desirable in flours to produce baked products or to be used as food additives (Awuchi et al., 2019). The microorganism used as starter culture for fermentation in this work was *Lactobacillus paracasei* (LP) CBA L74. Its probiotic nature and its ability to ferment cereal, fruit, and legume-based matrices were already studied in several works (Gallo et al., 2021a, b; Colucci Cante et al., 2021, Gallo et al., 2020a, b; Salameh et al., 2019). This work aimed at exploring the effect of optimizing mixing during a batch fermentation of a cooked navy bean suspension. Bacterial growth, lactic acid production, and sugar consumption were studied and compared with those observed in previous experimentations performed with a different mixing system (Colucci Cante et al., 2020). The resulting fermented products were characterized in terms of polyphenol and flavonoid content and antioxidant activity. Moreover, water adsorption capacity (WAC), water absorption index (WAI), oil absorption capacity (OAC), and foam capacity (FC) of the fermented freeze-dried powders were determined.

2. Materials and methods

Feedstocks and strain. LP CBA L74, patented and provided by Heinz Italia S.p.A, was used as starter culture. It was stored at -80 °C with glycerol (20 %) and reactivated through incubation at 37 °C for 24 h in 9 mL of an animal free broth (20 g/L Bacto Yeast Extract; Biosciences; 0.5 g/L MgSO₄, Sigma Aldrich; 50 g/L Glucose, Sigma Aldrich; 0.5 g/L Citric acid, Sigma Aldrich) before inoculation. Dried navy beans (Ontario crop) were provided by Heinz Italia S.p.A, Latina, Italy.

Fermentation apparatus and protocols. Pre-treatment and fermentation processes were carried out using the same experimental laboratory system and protocols described by Colucci Cante et al. (2020). Briefly, dried beans were soaked, cooked, and blended in a thermostatic mixer (Hot mix PRO TWIN-cutter). Fermentation apparatus consisted of a batch jacketed reactor equipped with an input for the insertion of a In Pro 3100 probe (Mettler Toledo), connected to a M300 transmitter (Mettler Toledo), useful for inline temperature/pH measurements. An impeller equipped with 3 Rushton turbines with different dimensions (I2) was designed for improving mixing during the process. Dried navy beans were soaked in deionized water (1:10 w/v) for 24 h at 25°C, cooked at 95°C for 2 h, and blended for 40 min. The resulting 1 L suspension was sterilized in autoclave at 134 °C for 40 min, cooled down to 37 °C, and inoculated (1% v/v, cell density of 10⁸ CFU/mL). Fermentation was carried out for 24 h, with a stirring rate of 81.4 rpm. During the process, bean samples were withdrawn aseptically at specific times (after the inoculum (t₀) and after 2 h (t₂), 4 h (t₄), 6 (t₆), 8 h (t₈), 14 h (t₁₄), 16 h (t₁₆), 18 h (t₁₈), 20 h (t₂₀), 22 h (t₂₂) and 24 h (t₂₄) of fermentation). For each sample, bacterial growth, lactic acid, sugar, and total starch were measured. Samples collected after cooking, at t₀ and t₂₄ (CB, FB₀, and FB₂₄, respectively) were freeze-dried and stored for further analysis. Doubling time, t_d, was calculated using Eq(1):

$$t_d = \frac{t_{exp}}{n} \quad (1)$$

where t_{exp} is the duration of the exponential phase and n is the number of cell generations, given by Eq(2):

$$N_f = N_0 * 2^n \quad (2)$$

where N₀ and N_f are the cell concentration (CFU/mL, where CFU are the colony forming units) at the beginning and at the end of the growth exponential phase, respectively.

Analytical methods. Decimal serial dilutions and a spread plate method on Petri plates filled with selective culture media were used to monitor bacterial growth. MRS agar (Oxoid) was used to assess LP CBA L74's

concentration; MacConkey agar (Oxoid) and Gelatine Peptone Bios Agar (Biolife) were used to control the presence of microbial contaminants in the fermenting medium. All plates were incubated at 37 °C for 48 h before reading. Lactic acid concentration was determined by high performance liquid chromatography (HPLC), Agilent Technologies 1100, equipped with an Agilent Zorbax C18 column (150 mm × 4.6 mm and a pore size of 8 µm) and a visible/UV detector. The eluent was 1 % NH₄H₂PO₄ aqueous solution at pH of 2.7, modified with H₂PO₄, with a flow rate of 0.8 mL/min. The detection was set at 218 nm. Fructose, glucose, and sucrose concentrations were determined by HPLC (Agilent Technologies 1100) with a Refractive Index (RI) detector, using a Rezex RHM-Monosaccharide column (300 mm × 7.8 mm and a pore size of 8 µm).

3.5 mM H₂SO₄ aqueous solution was eluted with a flow rate of 0.6 mL/min. Starch content was determined using a total starch assay kit (AA/AMG) (Megazyme). Extracts in ethanol solution (70 % w/v) of raw milled beans (RB), CB, FB₀, and FB₂₄ were prepared for total phenolic content (TPC) and total flavonoid content (TFC) analysis, according to the modified procedure reported by Aikpokpodion and Dongo, (2010).

0.5 g of powder were extracted with 7 mL of ethanol solution through sonication for 15 min. After centrifugation at 2000 rpm for 15 min, supernatant was recovered. The extraction procedure was repeated for 3 times and supernatants were combined. For TPC analysis, 1 mL of extract was mixed with 0.3 mL of Folin-Ciocalteu reagent, 1 mL of Na₂CO₃ (7.5 % w/v), and 8.7 mL of deionized water. After 2 h in the dark, absorbance was read at 720 nm using a spectrophotometer and deionized water was used as blank. Total phenolic amount was expressed as mg of gallic acid equivalent (GAE) through a calibration curve, previously prepared using gallic acid as standard. Flavonoid concentration was determined by aluminium chloride colorimetric method, reported by Ismail et al. (2017) with some modifications. 5 mL of each extract were mixed with 0.5 mL of 2 % w/v Al₂Cl₃ solution and 4.5 mL of 70 % ethanol. Each solution was shaken, and the absorbance was read at 420 nm after 2 h of incubation, using ethanol solution as blank. Total flavonoid concentration was indicated as mg of quercetin equivalent (QE), through a calibration curve obtained using quercetin as standard. Antioxidant activity (AA) of RB, CB, FB₀ and FB₂₄ extracts was evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) as free radical, according to the method reported by Brand-Williams et al. (1994). This spectrophotometric assay consisted of evaluating the DPPH scavenging activity of each extract. Solutions with different extract concentrations at fixed DPPH amount (4.47 × 10⁻⁵ M) were prepared. After 12 h, absorbances were read at 470 nm and the corresponding percent residual DPPH content, % [DPPH]_{res}, was calculated using Eq(3):

$$\% \text{ [DPPH]}_{\text{res}} = \frac{C - C_i}{C_i} * 100 \quad (3)$$

where C_i and C are the initial and the final DPPH concentrations, respectively, determined for each solution. AA of all the extracts was indicated as the amount necessary to reduce the initial DPPH content by 50 % (Efficient Concentration, EC₅₀). RB and FB₂₄ samples were characterized in terms of WAC, WAI, OHC, and FC. Analytical methods used by Sai-Ut et al. (2009) for their determination were adapted as described below. For WAC, WAI, and OHC evaluation, 1 g of sample was added to 10 mL of distilled water or olive oil for 1 min, stirred in vortex, and centrifuged at 5000 g for 30 min. Supernatant was recovered and its volume was recorded, using Eq(4) and (5) for calculation. For determining FC, 2 g of samples were weighed and added to 50 mL of distilled water, into a 100 mL graduated cylinder. The suspension was shaken and the total volume after 30 s was recorded. The percentage increase in volume after whipping was calculated using Eq(6):

$$\text{WAC or OHC} = \frac{V_{\text{initial water}} - V_{\text{supernatant}}}{m_{\text{weighed solid}}} \quad (4)$$

$$\text{WAI} = \frac{m_{\text{sedimented after centrifugation}}}{m_{\text{weighed solid}}} \quad (5)$$

$$\text{FC} = \frac{V_{\text{after whipping}} - V_{\text{before whipping}}}{V_{\text{after whipping}}} \times 100 \quad (6)$$

Statistical analysis was performed using Microsoft Excel 2016®. Mean values and standard deviations (n = 3) were calculated for each experimental data. Statistical significance was tested by Student's t-test at *p* < 0.05.

3. Results and discussion

Bacterial growth, lactic acid production, and sugar consumption curves determined with the mixing system I2 were compared with experimental data reported in Colucci Cante et al. (2020), as shown in Figure 1 and 2. Microbial growth evaluated with the optimized mixing system I2 started from an initial microbial charge of 3.84 × 10⁶ ± 3.58 × 10⁵ CFU/mL, immediately after inoculation, and showed a lag phase of 2 h.

Growth exponential phase lasted 12 h, against the 16 h of exponential period observed with I1. A maximum value of 1.01 × 10⁹ ± 2.05 × 10⁸ CFU/mL was reached at t₁₄ and remained constant until t₂₄.

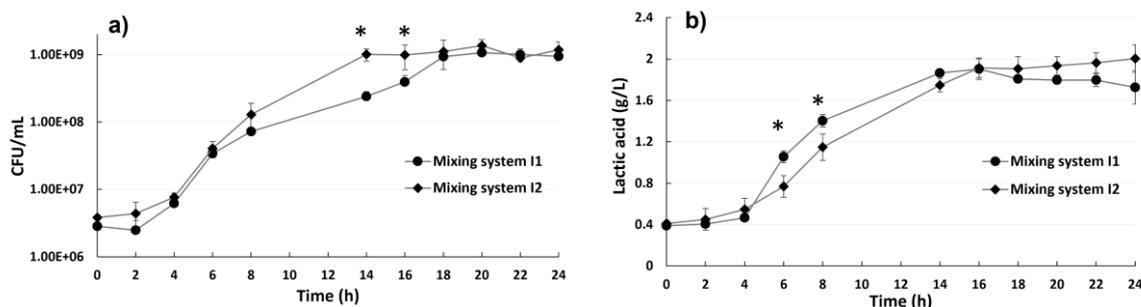


Figure 1: Comparison between (a) bacterial growth and (b) lactic acid production determined with impeller I1 (Colucci Cante et al., 2020) and the optimized impeller I2. $p < 0.05$ for all points marked with asterisks, *.

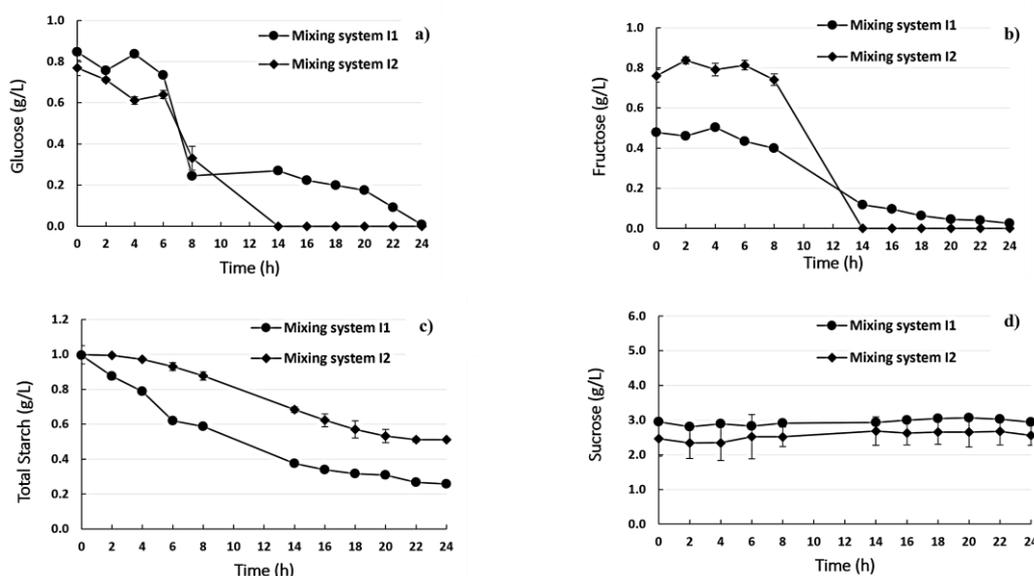


Figure 2: Comparison of (a) glucose, (b) fructose, (c) total starch, and (d) sucrose consumption during bean fermentation carried out with the old mixing system I1 (Colucci Cante et al., 2020) and the optimized I2.

Statistically significant differences between growth curves related to I1 and I2 were found at t_{14} and t_{16} , confirming the positive impact of improving mixing on the bacterial growth rate. By enhancing mass and heat transfer and reducing temperature and concentration gradients, a more homogeneous microenvironment for bacterial growth was ensured, as confirmed by Aguirre-Ezkauriatza et al. (2008) which studied the effect of mixing during fermentation in yogurt manufacturing. Lactic acid showed a growth-linked trend in both cases, but a slower increase was found with I2: a maximum value of approximately 2 g/L was reached at t_{16} and remained constant until the end of the process. When mixing was improved using I2, fermentation provided a lower doubling time, t_d (1.53 h), than that calculated with the old impeller I1 (1.87 h). The faster achievement of the stationary microbial load was due to a major availability of nutrients in the fermenting medium, as confirmed by the sugar trend in Figure 2. During the process performed with I2, glucose and fructose were rapidly and completely consumed by the microorganism during the exponential phase (Figure 2a and 2b), while total starch showed a milder decrease (from 0.998 ± 0.005 g/L to 0.512 ± 0.002 g/L) than that observed using I1 (Figure 2c), due to a greater availability of simple sugars for the microorganism when I2 was used. In both cases, sucrose remained approximately constant (Figure 2d). TPC content in RB (1.20 ± 0.05 mg GAE/g) remained approximately constant after cooking phase and was increased by the following sterilization treatment to a value of 2.77 ± 0.33 mg GAE/g in FB_0 (Table 1). Non-statistically significant differences were found between TPC values in FB_0 and FB_{24} . Probably, all the phenolic compounds covalently bound to the cell walls of several macromolecules, such as polysaccharides and proteins, were released during the autoclave treatment: fermentation process did not provide any effect on the amount of total free phenolic compounds. As for TPC, TFC in RB samples (0.13 ± 0.05 mg QE/g) seemed not to be changed by the cooking phase and a slight increase in FB_0 was observed due to the sterilization process (0.21 ± 0.04 mg QE/g): non-statistically significant changes after the fermentation process were observed. AA of the tested samples confirmed the

trend of TPC and TFC: a significant increase in DPPH scavenging activity, corresponding to an EC₅₀ reduction, was observed only in FB₀, after the autoclave treatment. In Nivedita and Sridhar (2014), beans were fermented with *Rhizopus oligosporus* immediately after cooking, without any further thermal treatment, and free polyphenols increased due to the fermentation. Conversely, in this work, the strong autoclave treatment applied before fermentation, was the main responsible for their release in the medium.

As shown in Table 1, bean fermentation led to a reduction of WAC and WAI, from values of 4.55 ± 0.03 mL/g and 5.53 ± 0.04 g/g, respectively, in RB samples, to 3.90 ± 0.14 mL/g and 4.31 ± 0.13 g/g, respectively, in FB₂₄. OHC decreased from 6.67 ± 0.12 mL/g to 3.11 ± 0.11 mL/g and FC reduced from 30 ± 1.65 % to 20 ± 1.30 % after 24 h of process. This slight WAC and WAI decrease observed was probably due to a reduction of soluble proteins during the fermentation process. Although an OHC increase is commonly associated with a decrease in WAC, due to exposure of non-polar amino acids or oil trapped on the surface after fermentation (Olukomaiya et al., 2020), the simultaneous reduction of both parameters observed in this work could be attributable on reaching a balance between hydrophilic-lipophilic groups of the compounds present through the fermentation process. Furthermore, FC decreased in fermented beans of 20 ± 1.30 % probably due to a partial proteolysis occurring during fermentation, as hypothesized by Xing et al. (2020) for leguminous flours.

Table 1: Total phenol and flavonoid content, efficient concentration, water and oil adsorption capacity, water adsorption index, and foaming capacity (TPC, TFC, EC₅₀, WAC, OHC, WAI, and FC, respectively) in raw, cooked, and fermented beans (RB, CB, FB₀ and FB₂₄, respectively). Values with different lowercase letters in the same column are significantly different ($p < 0.05$).

	TPC (mg GAE/g)	TFC (mg QE/g)	EC ₅₀ (mg/mL)	WAC (mL/g)	WAI (g/g)	OHC (mL/g)	FC (%)
RB	1.20 ± 0.05 ^a	0.13 ± 0.05 ^a	2.08 ± 0.08 ^a	4.55 ± 0.03 ^a	5.53 ± 0.04 ^a	6.67 ± 0.12 ^a	30 ± 1.65 ^a
CB	1.10 ± 0.18 ^a	0.16 ± 0.03 ^a	2.20 ± 0.15 ^a	-	-	-	-
FB ₀	2.77 ± 0.33 ^b	0.21 ± 0.04 ^b	1.50 ± 0.10 ^b	-	-	-	-
FB ₂₄	3.03 ± 0.54 ^b	0.20 ± 0.1 ^b	1.65 ± 0.18 ^b	3.90 ± 0.14 ^b	4.31 ± 0.13 ^b	3.11 ± 0.11 ^b	20 ± 1.30 ^b

4. Conclusions

Fermentation process of navy beans carried out using the probiotic strain LP CBA L74 was optimized to improve the bacterial growth kinetic and to evaluate the real potential of a leguminous matrix to produce semi-finished pro- and postbiotic products to be used in functional food formulation. The design of a new mixing system allowed to obtain a more homogeneous distribution of nutrients necessary for microorganism proliferation. A faster achievement of the maximum microbial load (1×10^9 CFU/mL at t_{14}) than that obtained with a different impeller used in a previous work was also confirmed by a lower doubling time value (1.53 h). Moreover, the effect of fermentation on the total polyphenol and flavonoid content was investigated, revealing that the sterilization process carried out before starting the fermentation could be considered the main responsible for their increase, from 1.20 ± 0.05 mg GAE/g and 0.13 ± 0.05 mg QE/g for polyphenols and flavonoids respectively, in RB, to the values of 2.77 ± 0.33 mg GAE/g and 0.24 ± 0.05 mg QE/g, for polyphenols and flavonoids respectively after sterilization. Antioxidant properties of the tested samples reflected the trend of TPC and TFC, showing an increase in DPPH scavenging activity exclusively due to the autoclave treatment performed before the fermentation. Moreover, the fermentation process led to a slight WAC and WAI decrease, probably due to a reduction of soluble proteins contained in the matrix, and to a significant decrease in oil retention and in foaming activity. Future purposes will be to maximize the concentration of other functional compounds, such as GABA, resistant starch, and serotonin and to investigate the LP's ability to reduce the content of undigestible oligosaccharides, such as raffinose and stachyose, phytates, and other antinutrients, responsible for several gut health diseases.

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