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Culture media based on effluent derived from soy protein concentrate production for *Lacticaseibacillus paracasei* 90 biomass production: statistical optimisation, mineral characterization, and metabolic activities

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Abstract The waste and by-products of the soybean industry could be an economic source of nutrients to satisfy the high nutritional demands for the cultivation of lactic acid bacteria. The aims of this work were to maximize the biomass production of *Lacticaseibacillus paracasei* 90 (L90) in three culture media formulated from an effluent derived from soy protein concentrate production and to assess the effects these media have on the enzymatic activity of L90, together with their influence on its fermentation profile in milk. The presence of essential minerals and fermentable carbohydrates (sucrose, raffinose, and stachyose) in the effluent was verified. L90 reached high levels of microbiological counts ($\sim$ 9 log cfu mL^{-1}) and dry weight ($>$ 1 g L^{-1}) on the three optimized media. Enzymatic activities (lactate dehydrogenase and β-galactosidase) of L90, and its metabolism of lactose and citric acid, as well as lactic acid and pyruvic acid production in milk, were modified depending on the growth media. The ability of the L90 to produce the key flavour compounds (diacetyl and acetoin) was maintained or improved by growing in the optimized media in comparison with MRS.

Keywords Culture medium · Lactobacilli · Industrial waste · Enzymatic activities · Fermented milk

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Introduction

In the last 20 years, the non-starter lactic acid bacteria (NSLAB) have been linked to the production of volatile compounds contributing to the flavour of most varieties of ripened cheeses. However, their presence has also been identified as responsible for some defects in cheese, as well as the accumulation of dangerous compounds, i.e. biogenic amines (Crow et al. 2001; Loizzo et al. 2013). Indeed, their effect is highly strain specific (Tanous et al. 2002; Pogačić

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et al. 2016; Stefanovic et al. 2017). Mesophilic lactobacilli, mainly *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum* and *Levilactobacillus brevis*, are the most often encountered and best studied NSLAB members (De Angelis et al. 2001). A large number of these strains have demonstrated adequate technological and biochemical properties for their use as adjunct cultures in the cheese industry (Crow et al. 2001).

Lactobacilli are nutritionally considered as very fastidious microorganisms, because of their nutritional requirements, including fermentable carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins, among others (Gänzle and Follador 2012). Over the years, numerous selective culture media have been developed for the growth of lactic acid bacteria (LAB), as well as MRS and Elliker, commonly used for lactobacilli and *Lactococcus*, respectively. Currently, commercial MRS medium, one of the most used in the world at laboratory scale, have a large number of components (dipotassium hydrogen phosphate, glucose, magnesium sulfate heptahydrate, manganous sulfate tetrahydrate, meat extract, peptone, sodium acetate trihydrate, triammonium citrate, yeast extract) which make lactobacilli grow very well. However, it is not economically viable for its use at industrial scale. The industrial production of lactobacilli strains requires complex culture media to fulfill its nutritional requirements, but also that these would be as simple as possible in order to be economical. The media used by culture suppliers for commercial production of concentrates are not public knowledge, though obviously they must satisfy the growth requirements of the organisms and must not hinder the concentration process (Parente et al. 2017).

A huge amount of waste and by-products from food industry are produced every year. Many of these have nutrients, including carbohydrates, peptides, amino acids, minerals and even vitamins, which could be used for lactobacilli culture production (Al Loman and Ju 2016; Ahmad et al. 2019; Chua and Liu 2019). Over the last two decades, several wastes have been proposed as culture media for lactobacilli, such as cheese whey, whey permeate (Fitzpatrick et al. 2001), tofu whey (Fei et al. 2018), and date juice (Nancib et al. 2001), among others. In particular, the production of soy protein concentrates (SPC) and soy protein isolates (SPI), used worldwide for food formulations, generates acid effluents, rich on carbohydrates (sucrose, raffinose, and stachyose), peptides and minerals (Coghetto et al. 2016). Coghetto et al. (2016) used this effluent to produce biomass of *Lactiplantibacillus plantarum* BL011 and lactic acid by its growing.

On the other hand, several studies have reported different levels of enzyme activities in LAB depending on the growth medium, especially on the type of carbohydrate and nitrogen source available (Vasiljevic and Jelen 2001; Zhong et al. 2018). Nowadays, there are many lactobacilli strains of NSLAB origin that have been characterized after growth in the MRS media like potential adjunct cultures because of their key enzyme activities. Also, there are many studies that reported good viability and biomass production for several strains in economic culture media derived from agro-industrial residues. However, there is little research about the evaluation of the enzymatic activity of the strains after growth in these alternative media.

Lactobacillus paracasei 90 (L90) (recently reclassified as *Lacticaseibacillus paracasei* 90) is a NSLAB origin strain that has shown a good performance as an adjunct in cheese: producing key compounds for the flavour development, accelerating proteolysis, as well as controlling cheese microflora. Also, L90 showed resistance to spray drying as a preservation process (Milesi et al. 2010; Peralta et al. 2017). All positive L90 studies have been performed after its growth in commercial MRS medium.

The aims of this work were to maximize the biomass production of *Lacticaseibacillus paracasei* 90 (L90) in three culture media formulated from an effluent derived from soy protein concentrate production and to assess the effects these media have on the enzymatic activity of L90, together with their influence on the fermentation profile of this strain in milk.

Materials and methods

Bacterial strain

L90 (INLAIN collection) was stored frozen at − 80 °C in MRS broth with 15% (v/v) of glycerol and it was routinely grown in MRS broth at 34 °C for 18 h.

Base culture medium (BCM)

Soy protein concentrate was produced according to Wang et al. (2004). Soybean meal was homogenised (12,000 rpm for 5 min) with distilled water (10% w/v) in an Ultraturrax Ika T25 Digital homogeniser (IKA Works, Germany). Then, the mixture was acidified with 2 M HCl until reaching pH = 4.5 (Roopashri and Varadaraj 2014). The mixture was kept under stirring at 40 °C for 30 min to facilitate the extraction of the soluble compounds. Then, it was centrifuged (5000 rpm for 20 min at 4 °C) to obtain the protein/fiber (solid part) and the effluent (liquid part). This effluent was used to prepare a base culture medium (BCM) for the growth of L90.

The pH of the liquid part was adjusted to 8 by dropwise addition of 8 M NaOH and then heated at 115 °C for 10 min. After cooling to room temperature, the mixture was centrifuged (5000 rpm for 20 min at 4 °C). Finally, the pH of the supernatant was adjusted to 6.5 by dropwise addition of 2 M HCl and sterilized in an autoclave (15 min at 121 °C). This sterilized liquid was used as the BCM.

Optimization

Response surface methodology (RSM) with a central composite design (CCD) was used to maximise the production of L90 biomass in three culture media formulated from BCM added with some nutrients, described below. The factors (glucose, lactose, yeast extract, $MnSO_4$ and $MgSO_4$) and its concentrations were defined according to a preliminary screening for L90 and to the optimization reported for *Lactiplantibacillus plantarum* BL011 in a similar effluent (Coghetto et al. 2016). Table S1 shows the factors of the three designs studied (D1, D2 and D3), which were differenced by the carbon source employed. Glucose and lactose were studied in D1 and D2, respectively, while D3 only had the base medium carbohydrates. The addition of lactose in D2 was made by adding cheese whey permeate powder (86% w/w lactose), which is an important by-product of many Argentinian industries. On the other hand, yeast extract, $MnSO_4$ and $MgSO_4$ were included in the three designs. An amount of 22 experimental runs with 6 central points were made for each design, and these were randomized and divided in two blocks. The complete matrixes of the three experimental designs are shown in Table S2 in Supplementary material. For each run, 100 mL of the culture medium were prepared; the same BCM was used for all runs in each design. L90 was inoculated at 2% (v/v) in each medium and incubated in a thermostated water bath at 34 °C for 24 h. As controls, L90 was inoculated and incubated in the same conditions in MRS and BCM. As biomass measure of L90 (response), microbiological plate counts (MC) and dry cell weight (DW) were carried out at the end of the incubation. The experimental results were analysed by fitting to mathematical models, which were statistically validated by ANOVA and lack-of-fit test to evaluate the significance of each model. Response surface graphs were analysed and the desirability function was used to find optimum concentrations of each factor to maximize the biomass production (MC and DW). Finally, the models were validated under the predicted conditions by triplicate. The optimized culture media obtained from the D1, D2 and D3 designs were named as M1, M2 and M3, respectively.

Biomass analysis

For microbiological counts (MC), serial dilutions of the suspensions were prepared using 0.1% (w/v) sterile peptone water. L90 was enumerated on MRS agar after microaerobic incubation for 48 h at 37 °C. Dry weight (DW) of cells was gravimetrically measured according to Coghetto et al. (2016). A volume of 40 mL of each cellular suspension was transferred into preweighed 50-mL Falcon tube, centrifuged ($10{,}000 \times g$ for 15 min at 4 °C) and washed twice with cold distilled water. Finally, they were dried at 80 °C to constant weight (20–24 h).

pH in culture media

Measurement of pH was made immediately after completion of the fermentation using a pH meter (Orion Research Inc. USA).

Carbohydrates and organic acids in culture media

Carbohydrates and organic acids in the culture media after incubation were identified and quantified by HPLC according to Peralta et al. (2014). An Aminex HPX-87H column (300 × 7.8 mm) held at 32 °C, with a cation H + microguard cartridge (Bio-Rad

Laboratories, USA) was used for the separation. An UV, set at 210 nm, and a refractive index detectors in series were used to detect organic acids and carbohydrates, respectively. Analysis was performed isocratically at 0.6 mL min^{-1} using 10 mM H_2SO_4 as the mobile phase.

For the analytes that eluted independently (lactic acid, acetic acid, lactose), best-fit standard curves were made by linear regression of peak area vs. concentration of the analyte. The concentration of the coeluting analytes: stachyose + raffinose (coelution present in all optimized media) and lactose + sucrose (coelution present in M2), was estimated with standard curves of raffinose and sucrose, respectively.

Samples were diluted 1:3 in the mobile phase, and filtered through PVDF filters of 0.45 μm pore size (Millipore, São Paulo, Brazil) immediately before the HPLC analysis.

Mineral and trace elements composition

The samples were acidified with ultrapure nitric acid (Sigma Aldrich, Missouri, USA) at 1% (v/v) and were stored at 2 °C until analysis. The ultrapure nitric acid was double-distilled using an infra-red subboiling distillatory system (BSB-939-IR, Berghof, Germany) prior to use. Mineral and trace element composition (Mg, Mn, Ca, Na, Fe, Zn, Ti, Al, Cr, Cu, Ba, Co, Mo, Sr, Ni, Pb, Ag, Ti, V, Sn, Sb, and Be) was performed using an inductively coupled plasma mass spectrometer (ICP-MS, Elan 9000, Perkin Elmer Life and Analytical Sciences, Shelton, USA). For data acquisition, a Perkin Elmer ELAN 3.5 software was used. Limit of quantitation of each mineral and trace element is shown in Table 3.

Enzymatic activities

Lactate dehydrogenase (LDH) and β-galactosidase (β-GAL) activities were determined in cell-free extracts of L90 after growth in each optimized medium and in MRS. Cell-free extracts (CFE) were obtained according to Peralta et al. (2016a). Cells were harvested from 50 mL of cell culture media by centrifugation (10,000×*g* for 10 min at 4 °C), washed twice with 50 mM potassium phosphate buffer, pH 7, and then resuspended in an appropriate volume of the same buffer containing glass beads (1.2 g) (106 μm, Sigma) to give a 30-fold concentration of the cells. Samples were homogenised for 3 min at maximum speed in a Minibeadbeater 8TM cell disrupter (Biospec Products Bartlesville, OK, USA) and finally centrifuged (16,000×*g* for 15 min at 10 °C). After centrifugation, the beads, cells, and cell debris were discarded and the supernatants were filtered through a 0.45 μm pore diameter membrane and kept frozen until the enzymatic analysis.

LDH and β-GAL activities were assessed according to Daryaei et al. (2010) and Vinderola and Reinheimer (2003), respectively. Cell-free extracts obtained from the cells grown in M1, M2, M3 and MRS were identified as CFEm1, CFEm2, CFEm3 and CFEmrs, respectively.

Milk fermentation

L90 cells from M1, M2, M3 and MRS were inoculated at 2% (v/v) in 250 mL of UHT whole milk, fractionated in sterilized tubes and incubated at 37 °C for 7 days. The values of pH were determined at 0, 1, 3, 5 and 24 h of incubation. Microbiological analysis, proteolytic activity, carbohydrates and organic acids were assessed after 24 h of incubation, while diacetyl and acetoin were assessed at 24 h and 7 day of incubation. In parallel, non-inoculated milk was incubated as milk control (NIM). Fermented milks (FM) inoculated with cells from M1, M2, M3 and MRS were identified as FMm1, FMm2, FMm3 and FMmrs, respectively.

pH, microbiological analysis and proteolytic activity in fermented milk

The determination of pH and microbiological counts in the milk samples were made as described above. Proteolytic activity was determined by the o-phthaldialdehyde (OPA) spectrophotometric assay. The levels of proteolytic activity were expressed as the difference in absorbance at 340 nm between the fermented milk and non-inoculated milk (ΔAbs_{340nm}).

Carbohydrates and organic acids in fermented milk

Carbohydrates (lactose) and organic acids (citric, orotic, hippuric, pyruvic, lactic and acetic acids) in fermented milk were analysed by HPLC as detailed above but the chromatography column was held at 65 °C instead 32 °C. Samples were diluted 1:10 in the

Table 1 Mathematical models

Design	Response[1]	Model	p value	Lack of fit	Equations[4]
D1	MC	Modified cubic[2]	0.0074	0.5387	$MC_1 = 8.2 + 1.6B + 44.6D - 1.8A_1B + 67.0BC + 361.0D^2 + 98.4A_1BD$
	DW	Quadratic	< 0.0001	0.8435	$DW_1 = 0.95A_1 + 2.35B - 0.29A_1^2 - 1.48B^2 - 328.82D^2$
D2	MC	Modified cubic[2]	0.0358	0.8317	$MC_2 = 10.07 - 1.15B - 0.12A_2^2$
	DW	Quadratic	< 0.0001	0.9988	$DW_2 = 0.68 + 0.37A_2 + 1.19B + 67.97C - 8.18D + 0.26A_2B - 21.02A_2C + 9.30BD - 0.09A_2^2 - 0.86B^2 - 4830.94C^2 + 114.10D^2$
D3	MC	2FI[3]	0.0072	0.9787	$MC_3 = 9.74 - 27.22C + 22.91BD$
	DW	Linear	< 0.0001	0.1706	$DW_3 = 0.66 + 0.62B$

[1]*MC* microbiological counts, *DW* dry weight

[2]Modified cubic model: the cubic term was removed by backward multiple regression method (Myers and Montgomery 2009)

[3]2FI: first-order model, interaction between two factors

[4]A_1: glucose; A_2: lactose; B: yeast extract; C: $MnSO_4$; D: $MgSO_4$

mobile phase, homogenised, centrifuged (15,000×*g* for 20 min at 4 °C) and finally filtered through a 0.45 µm membrane immediately before of the HPLC analysis.

Diacetyl and acetoin in fermented milk

Diacetyl and acetoin were analysed by SPME-GC-FID/MS according to Peralta et al. (2017). The samples of fermented milk (10 g) were transferred into GC vials and were heated to 45 ± 1 °C for 10 min; then the fiber CAR/PDMS 75 µm (Supelco Inc. Bellefonte, PA, USA) was directly exposed to the vial headspace for 30 min. The gas chromatography system (Perkin Elmer model 9000, USA) was equipped with a HP INNOWax column (60 m × 0.25 mm × 0.25 µm) from Agilent Technologies. The oven temperature program was set as follows: 45 °C for 5 min, the temperature increased to 150 °C for 3 min at a ramp of 8 °C min^{-1} and, finally, increased to 250 °C for 5 min at a ramp of 10 °C min^{-1}. A FID detector, set at 290 °C, and hydrogen as the carrier gas, at a flow rate of 2.0 mL min^{-1}, were used. Peak areas were expressed in arbitrary units (a.u. = peak area × 10^{-3}).

Statistical analysis

Experimental designs and its statistical analysis were made with the Design-Expert software version 11 (free trial). The models were obtained for each response with backward regression method (Myers and Montgomery 2009). One-way analysis of variance (ANOVA) and post-hoc Tukey test were applied to compare the means of enzymatic activities and all variables studied in the media composition validation and milk fermentation with Statgraphics Centurion XVI software (free trial). Data were considered significantly different when $p < 0.05$. The R packages ggplot2 was used for visualizing the connection between variables and between samples in a heatmap made with Z-scores values. The colour scale represents the abundance of each variable, denoted as Z-score, with red indicating high abundance and blue indicating low abundance.

Results

Optimization

Microbiological counts (MC) and dry weight (DW) of each run in the three experimental designs are shown in Table S2. Both responses were fit to mathematical models according to the equations shown in Table 1. In these equations are presented only the significant terms ($p < 0.05$). The p-values for the six models were significant ($p < 0.05$). On the other hand, the lack of fit of the six models were non-significant ($p > 0.05$),

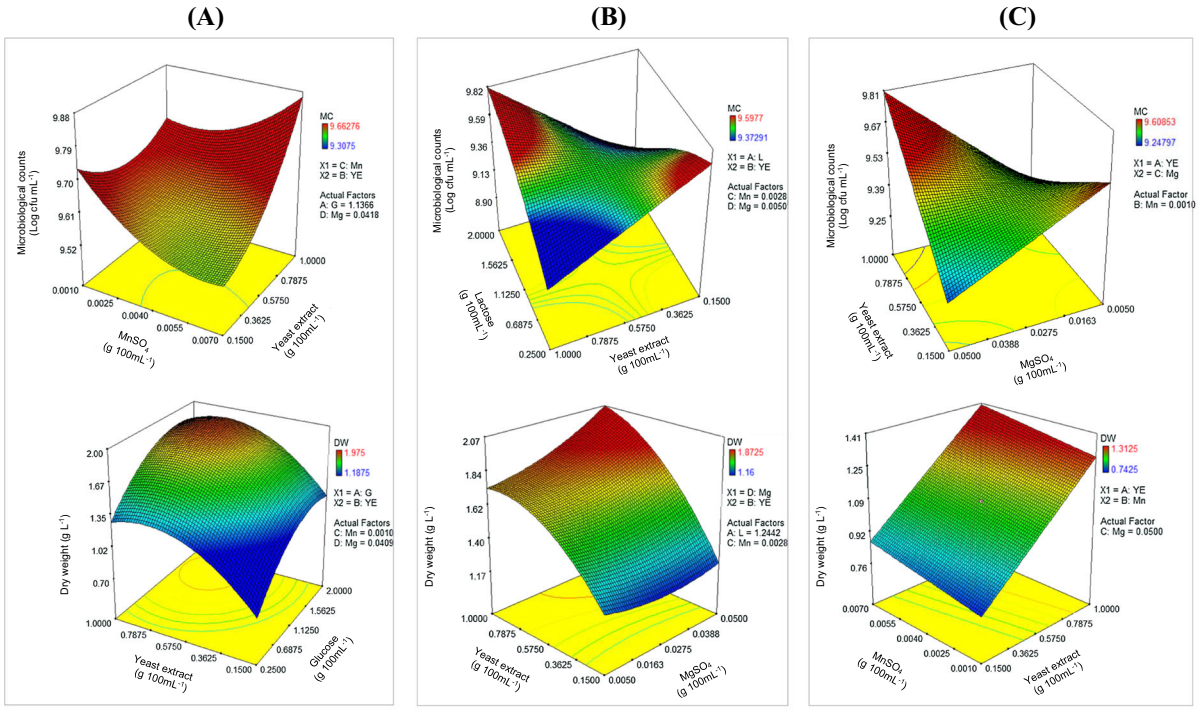


Fig. 1 Response surface of the effect of the main factors on microbiological counts and dry weight in the design 1 (**A**), design 2 (**B**) and design 3 (**C**)

indicating that the models are suitable to describe the responses.

Response surfaces with most significant factors for each model (D1, D2 and D3) were graphed (Fig. 1). For D1, highest levels of MC could be occurred at highest yeast extract and $MnSO_4$ concentrations. However, high levels of MC may also be obtained by decreasing both factors. For D2, high levels of MC could be occurred at low concentrations of both yeast extract and lactose, while high levels of DW could be occurred at high yeast extract and $MgSO_4$ concentrations. Finally, for D3 high MC might be obtained at high concentrations of $MgSO_4$ and yeast extract. In addition, yeast extract is essential to increase the DW in D3.

Table 2 shows the concentrations of the factors for each optimized media and its predicted values. These compositions were validated by triplicate.

L90 reached high levels of MC and DW on the three optimized media: $> 10^9$ cfu mL^{-1} and > 1 g L^{-1}, respectively (Fig. 2A). Dry weight in M1, M2, M3 and MRS was 3.6, 3.7, 2.8, and 6.7-fold higher than in base culture medium (BCM), respectively. These results showed a good agreement between predicted and experimental responses, and implied that the mathematical models were suitable for the simulation of biomass production.

The levels of carbohydrates, lactic acid, acetic acid and pH in the optimized media before and after incubation are shown in Fig. 2B, C.

The carbohydrates detected in the optimized media were not the same since these had a different composition (Table 2). Glucose was present in all media at different levels: low in M2 and M3 and high in M1 and MRS because this sugar was added as a nutrient in these last two media. On other hand, the carbohydrates commonly present in soy derivatives (stachyose, raffinose and sucrose), there were in the three optimized media (M1, M2 and M3). Stachyose and raffinose were overlapped in these samples, so these carbohydrates could not be differentiated. In this same way, the sucrose in the M2 sample was overlapped with the added lactose, and therefore neither was possible to quantify individually these carbohydrates.

Table 2 Optimized media composition and predicted responses under the central composite design

Optimized media	Factor					Predicted responses	
	Glucose (g 100 mL^{-1})	Lactose (g 100 mL^{-1})	Yeast extract (g 100 mL^{-1})	$MnSO_4$ (g 100 mL^{-1})	$MgSO_4$ (g 100 mL^{-1})	MC[1] (log cfu mL^{-1})	DW[2] (g L^{-1})
M1	1.1366	–	0.5329	0.0010	0.0418	9.66	1.73
M2	–	1.2442	0.5716	0.0028	0.0500	9.53	1.80
M3	–	–	0.6823	0.0011	0.0500	9.61	1.10

[1]*MC* microbiological counts

[2]*DW* dry weight

The levels of glucose were significantly reduced ($p < 0.05$) in M1 and MRS; in the last media, this sugar was totally consumed. The rest of the carbohydrates (raffinose, sucrose, and stachyose) in M1 were not consumed by L90. In M2, a significant ($p < 0.05$) reduction of the levels of the peak corresponding to the overlapped carbohydrates (sucrose and lactose) was observed. However, it was not possible to identify which of them decreased because of the coelution mentioned above. Finally, a decrease of sucrose was observed in M3.

The mean values of lactic acid were between 20 to 31 mg 100 mL^{-1}, while the levels of acetic acid were lower (between 0.1 to 5.8 mg 100 mL^{-1}). The lowest and highest lactic acid production were observed in M3 and MRS ($p < 0.05$), respectively. The pH values were lower in M1 (3.65) and M2 (3.85) than in M3 (4.12) and MRS (4.07).

Mineral and trace elements composition

The mineral and trace elements composition in the base culture medium (BCM), all optimized media (M1, M2, M3) and MRS are shown in Table 3. Sixteen minerals were detected in the culture media; nine of them (Mg, Mn, Ca, Zn, Ti, Cr, Cu, Mo and Ni) presented significant differences between media. High levels (16–328 mg L^{-1}) of Na, Mg and Ca and low levels (1.52–0.01 mg L^{-1}) of Mn, Zn, Fe, Ti, Al, Cr, Cu, Ba, Co, Mo, Sr, Ni and Pb were found in BCM. The concentration of Mg, Cu, Mo and Ni was significantly higher in BCM compared to MRS. In addition, the levels of Mn and Cu were numerically higher in M2 in comparison to the other media derived from soy (BCM, M1, and M3). As expected, the levels of Mg and Mn in all optimized media were higher than BCM. On the other hand, the concentration of Mn, Ti and Cr was significantly higher in MRS compared to the other media.

Enzymatic activities

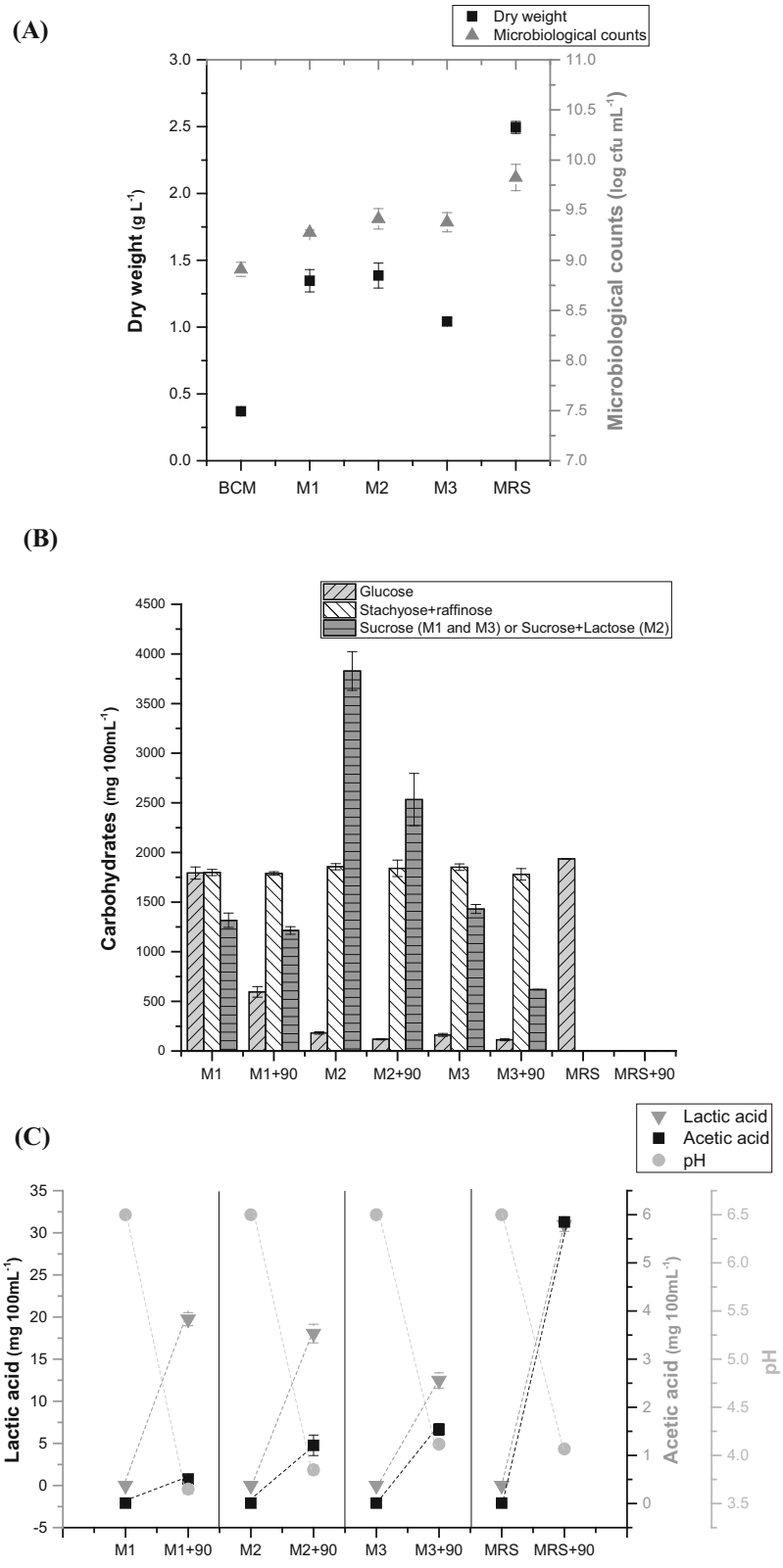
LDH and β-GAL levels are shown in Fig. 3A, B, respectively**.** The mean levels of LDH were between 0.31 and 0.55 nmol NADH min^{-1} μL^{-1} extract, while the levels of β-GAL were lower (between 0.020 and 0.041 nmol ONP min^{-1} μL^{-1} extract). LDH activity did not significantly differ between extracts ($p > 0.05$); however, the levels in CFEmrs were numerically higher in comparison with the other extracts. The levels of β-GAL in CFEm1 were significantly lower ($p < 0.05$) than the levels observed in CFEmrs, while in CFEm2 and CFEm3 the levels were intermediate but without significant differences.

Milk fermentation

pH, microbiological counts and proteolytic activity

pH values and microbiological counts are shown in Fig. 4A, B, respectively. Milk fermented with the cells grown in MRS (FMmrs) presented lower pH values ($p < 0.05$) in comparison with the other fermented milks at 24 h of incubation. Regardless of the growth medium, L90 reached 9 log cfu mL^{-1} at 24 h of incubation, and no significant differences were found between fermented milks.

The statistical analysis of the proteolytic activity revealed no significant differences between fermented milks; in addition, the obtained values were low (Fig. 4B).


(A)
Dry weight
Microbiological counts
Dry weight (g L-1)
Microbiological counts (log cfu mL-1)
BCM
M1
M2
M3
MRS
(B)
Glucose
Stachyose+raffinose
Sucrose (M1 and M3) or Sucrose+Lactose (M2)
Carbohydrates (mg 100mL-1)
M1
M1+90
M2
M2+90
M3
M3+90
MRS
MRS+90
(C)
Lactic acid
Acetic acid
pH
Lactic acid (mg 100mL-1)
Acetic acid (mg 100mL-1)
pH

◀**Fig. 2** **A** Biomass of L90 (dry weight and microbiological counts) in the three optimized media (M1, M2 and M3) during validation, and in the commercial medium for growth of lactobacilli (MRS) and the base culture medium (BCM). **B** Carbohydrates and **C** lactic acid, acetic acid and pH of the three optimized media (M1, M2 and M3) and MRS

Lactose and organic acids

Lactose and organic acids in milks after 24 h of incubation are shown in Fig. 4C. Lactose, citric acid, orotic acid, and hippuric acid concentrations decreased significantly ($p < 0.05$) in all fermented milks. The greatest decrease of lactose was observed in FMmrs while the smallest reduction was detected in FMm1. Citric acid was completely consumed in FMm1, FMm3 and FMmrs, while a partial consumption was observed in FMm2. The concentration of orotic acid in FMm1 was higher ($p < 0.05$) than in the other fermented milks ($p < 0.05$). Hippuric acid decreased entirely in all fermented milks.

On the other hand, a production of lactic, pyruvic and acetic acids was detected. The highest and lowest levels of lactic acid were observed in FMmrs and FMm1, respectively, while in FMm2 and FMm3 the concentrations were intermediate.

Table 3 Minerals and trace elements composition

Minerals[1]	LOQ[2] (mg L^{-1})	BCM (mg L^{-1})	M1 (mg L^{-1})	M2 (mg L^{-1})	M3 (mg L^{-1})	MRS (mg L^{-1})	Level of significance[3]
Mg	0.1	116.3 ± 8.7^{b}	$140.9 \pm 19.5^{b,a}$	157.1 ± 4.0^{a}	156.6 ± 19.6^{a}	33.1 ± 2.3^{c}	**
Mn	0.001	0.167 ± 0.021^{c}	$1.835 \pm 0.025^{b,c}$	3.573 ± 0.975^{b}	$2.187 \pm 0.225^{b,c}$	10.323 ± 1.046^{a}	**
Na	0.1	328.0 ± 23.4	212.2 ± 152.2	539.9 ± 270.7	337.9 ± 32.1	364.6 ± 32.4	n.s
Ca	0.1	$16.0 \pm 0.3^{a,b}$	27.2 ± 17.1^{a}	26.8 ± 0.3^{a}	$17.3 \pm 2.0^{a,b}$	5.4 ± 1.6^{b}	*
Zn	0.001	$1.517 \pm 0.101^{b,c}$	$1.943 \pm 0.244^{a,b}$	$1.860 \pm 0.046^{a,b}$	2.140 ± 0.234^{a}	1.297 ± 0.131^{c}	**
Fe	0.01	0.73 ± 0.05	0.99 ± 0.29	0.99 ± 0.08	1.05 ± 0.33	1.08 ± 0.16	n.s
Ti	0.01	0.19 ± 0.02^{c}	$0.34 \pm 0.05^{c,b}$	0.48 ± 0.01^{b}	$0.39 \pm 0.03^{c,b}$	1.67 ± 0.22^{a}	**
Al	0.01	0.18 ± 0.13	0.14 ± 0.03	0.17 ± 0.04	0.15 ± 0.03	0.20 ± 0.04	n.s
Cr	0.001	0.050 ± 0.000^{c}	0.080 ± 0.010^{b}	0.083 ± 0.006^{b}	$0.067 \pm 0.006^{b,c}$	0.110 ± 0.010^{a}	**
Cu	0.001	0.257 ± 0.025^{a}	0.250 ± 0.056^{a}	0.293 ± 0.015^{a}	0.263 ± 0.047^{a}	0.073 ± 0.023^{b}	**
Ba	0.001	0.020 ± 0.010	0.043 ± 0.049	0.013 ± 0.006	0.020 ± 0.000	0.037 ± 0.021	n.s
Co	0.001	0.010 ± 0.000	0.013 ± 0.006	0.010 ± 0.000	0.017 ± 0.006	0.017 ± 0.012	n.s
Mo	0.001	0.300 ± 0.020^{a}	0.283 ± 0.032^{a}	0.290 ± 0.010^{a}	0.303 ± 0.029^{a}	0.010 ± 0.000^{b}	**
Sr	0.001	0.073 ± 0.006	0.147 ± 0.116	0.090 ± 0.010	0.080 ± 0.000	0.043 ± 0.012	n.s
Ni	0.001	0.207 ± 0.015^{a}	0.200 ± 0.026^{a}	0.197 ± 0.006^{a}	0.210 ± 0.017^{a}	0.010 ± 0.000^{b}	**
Pb	0.001	0.113 ± 0.097	0.137 ± 0.090	0.110 ± 0.098	0.090 ± 0.087	0.100 ± 0.096	n.s
Ag	0.001	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
Tl	0.001	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
V	0.01	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
Sn	0.001	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
Sb	0.001	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
Be	0.001	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	

[1]Values presented are means ± standard deviation of the triplicate independent formulation of media

[2]LOQ: Limit of quantitation

[3]*$p < 0.05$; **$p < 0.001$; n.s. no significant differences

Base culture medium (BCM); Optimized media (M1, M2 and M3); Commercial medium for growth of lactobacilli (MRS); see Material and methods for details

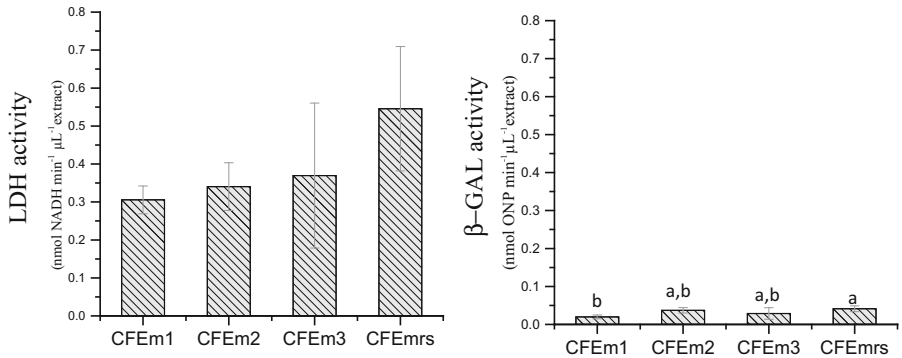


Fig. 3 **A** Lactate dehydrogenase (LDH) activity. **B** β-galactosidase (β-GAL) activity. Different letters indicate significant differences according to Tukey test ($p < 0.05$)

Pyruvic acid production was lower in FMmrs ($p < 0.05$) in comparison with the other fermented milks, while the levels of acetic acid were similar in all fermented milks.

Diacetyl and acetoin

The production of both volatile compounds, diacetyl and acetoin, had a similar trend in each fermented milk (Fig. 5). The level of diacetyl in FMm2 after 24 h of incubation was significantly higher ($p < 0.05$) than in FMmrs and in the non-inoculated milk (NIM), while the values were intermediate in FMm1 and FMm3. Similarly, the level of acetoin in FMm2 after 24 h of incubation was significantly higher than in the other fermented milks ($p < 0.05$), while there were not significant differences between FMmrs, FMm1 and FMm3. After 7 days of incubation, the levels of both compounds had similar trends than that observed at 24 h. Diacetyl levels decreased in all cases, practically to the half of the initial values, while acetoin levels were slightly lower than those observed after 24 h of incubation. For both compounds, FMm2 showed significantly higher levels than the rest of the fermented milks ($p < 0.05$), while FMmrs did not vary significantly respect to the NIM ($p > 0.05$).

Furthermore, a multivariate analysis using a heat map and clustering method was performed to find statistically significant relationships between the results for diacetyl and acetoin and the other variables (pH, organic acids, enzymatic activities and lactobacilli counts) after 24 h of incubation (upper dendrogram), as well as, relationships between fermented milks (left dendrogram) (Fig. 6). The left dendrogram shows that samples were divided into three clusters according to the media where L90 was grown: M2 (cluster 1), M1 and M3 (cluster 2) and MRS (cluster 3). In the upper dendrogram, an association between some variables could be observed. In this sense, orotic acid, pH, citric acid and pyruvic acid were grouped in cluster 4 together with acetoin and diacetyl. Besides, lactic acid and lactobacilli counts were grouped in cluster 5, together with LDH and β-GAL.

Discussion

Over the years, a large number of culture media have been proposed for the LAB growth such as MRS and M17, which are the most commonly used. Although these media generally produce high biomass levels, they are rarely used to produce bacteria at scale up by they are unprofitable and so, these are mainly limited to quality control, laboratory analysis, research studies, and academic purposes (Hayek et al. 2019). Consequently, industries are actively searching for low cost products that can replace expensive components and support the growth and cell mass production of LAB.

In this work, we maximized the biomass production of L90 in three culture media formulated from the effluent of the production of soy protein concentrates, putting attention on the influence of the growth media on the enzymatic and metabolic activity of the strain. This effluent, used as BCM in the present work, as well as other by-products and residues of soybean

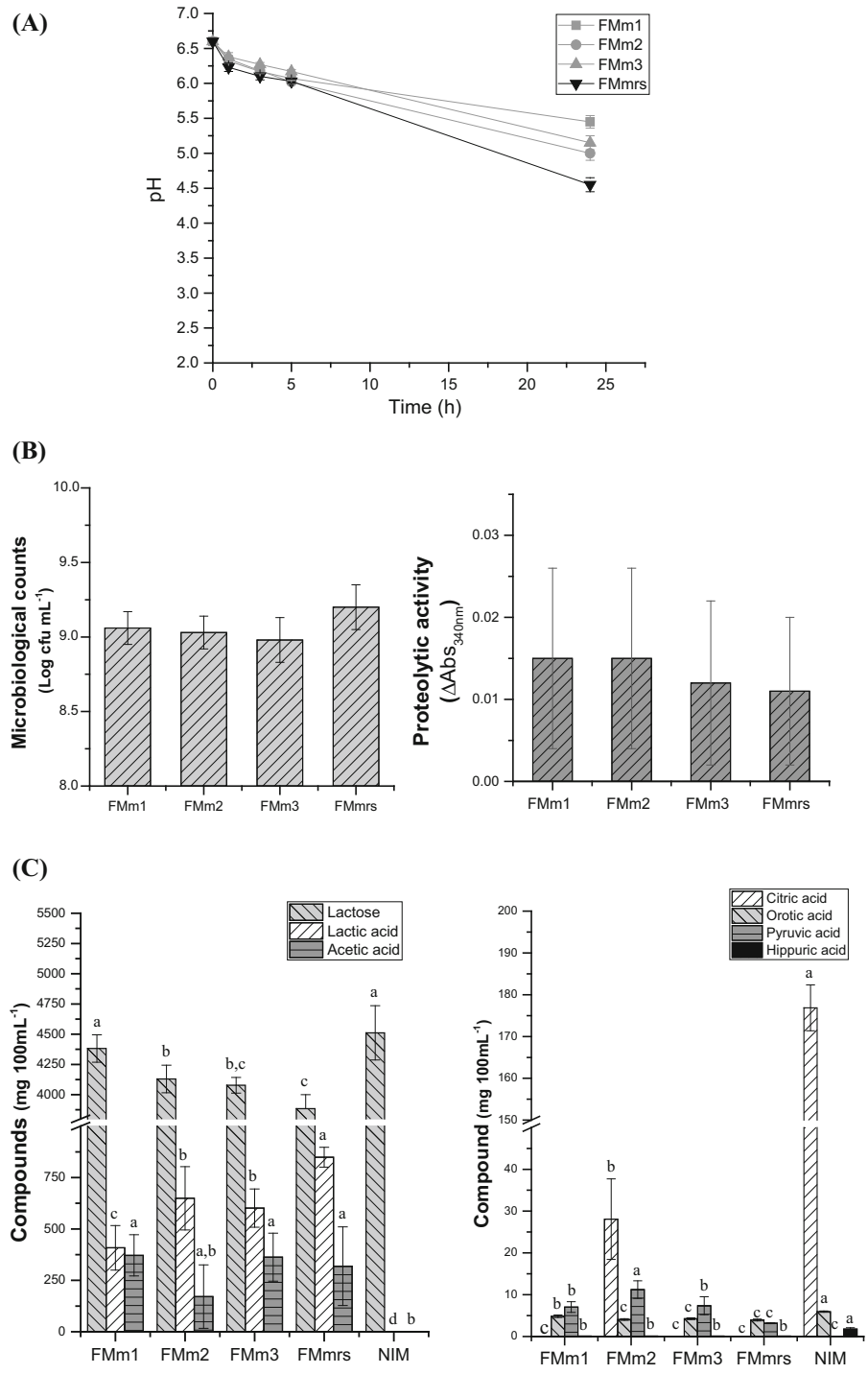


Fig. 4 Results of pH during incubation (**A**), and microbiological counts, proteolytic activity (**B**), and levels of lactose and organic acids after 24 h of incubation (**C**) in fermented milks (FM) inoculated with L90 grown in optimized media (M1, M2 and M3) and MRS; NIM: non-inoculated milk. Different letters for the same compound indicate significant differences according to Tukey test ($p < 0.05$)

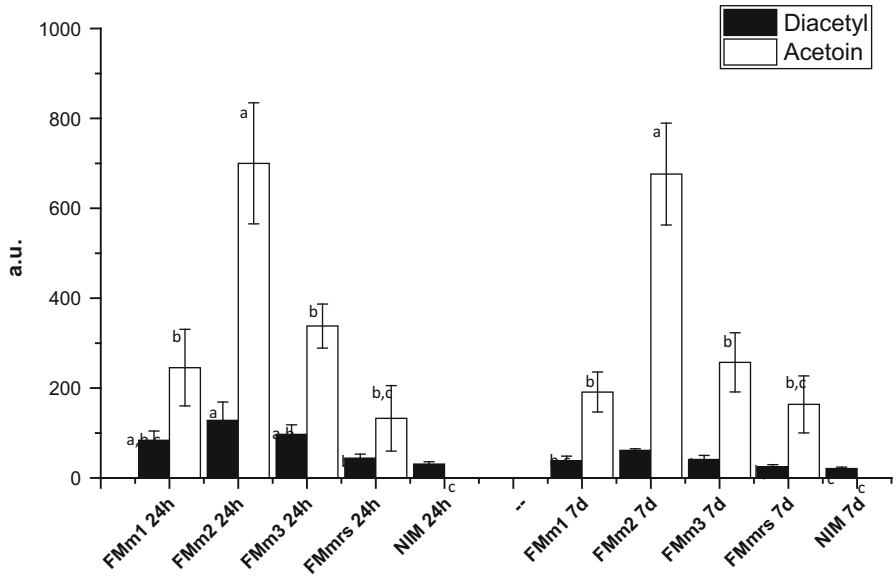


Fig. 5 Levels of diacetyl and acetoin in fermented milks (FM) inoculated with L90 grown in optimized media (M1, M2 and M3) and MRS; NIM: non-inoculated milk. For each time of incubation, different letters for the same compound indicate significant differences according to Tukey test ($p < 0.05$)

processing (soybean hulls, soybean meal, soybean okara, soybean molasses) have many soluble nutrients, including carbohydrates, minerals, amino acids and peptides, among others (Al Loman and Ju 2016; Chua and Liu 2019), which could be used by L90 for their growth. In our work, we verified the presence of essential minerals for the growth of bacteria and fermentable carbohydrates (sucrose, raffinose, and stachyose) in BCM. This would explain the ability of L90 to grow in the BCM, reaching 8 log cfu mL^{-1}. Although we observed that L90 grew in this basal medium, it was necessary to incorporate other components in order to comply with industrial requirements (levels $> 1 \times 10^9$ cfu mL^{-1}) (Tamime et al. 2006).

Most LAB species, as lactobacilli, prefer glucose as energy source (Lu et al. 2001; Andreevskaya et al. 2016). That is the reason why the MRS, the main culture medium for lactobacilli growth, has glucose among its components, and also explains that its incorporation into M1 has improved the response in the model. However, other sources of energy could be used by LAB (Lu et al. 2001). The ability to use the main carbohydrates of soybean (sucrose, raffinose and stachyose) and its derivatives (Romão et al. 2010) has been reported in many LAB, including *Lacticaseibacillus paracasei*, *Limosilactobacillus fermentum*, *Limosilactobacillus reuteri*, *Lactiplantibacillus plantarum*, *Lactobacillus amylolyticus* and *Lactobacillus acidophilus*, as well as in *Bifidobacterium*, including *B. animalis* and *B. longum* (Desai et al. 2002; Lê et al. 2003; Gänzle and Follador 2012; Fei et al. 2018; Zartl et al. 2018). In particular, the consumption of these carbohydrates by the specie *Lacticaseibacillus paracasei* was reported by Lê et al. (2003) and more recently by Zartl et al. (2018). Lê et al. (2003) observed that the strain *Lacticaseibacillus paracasei* LG3 grew well ($\sim$ 8 log) in a liquid by-product of the tofu production that contain sucrose, raffinose, and stachyose as main carbohydrates. The authors noted that only the sucrose was consumed by *Lacticaseibacillus paracasei* LG3; as well as we found for L90 in M3, which only had the carbohydrates of the BCM. On the other hand, Zartl et al. (2018) observed that the consumption of stachyose and raffinose by strains of lactobacilli, including *Lacticaseibacillus paracasei,* was strain-dependent: *Lacticaseibacillus paracasei* DSM 20312 grew well with these sugars, while *Lacticaseibacillus paracasei* CRL431 was not able to grow with either of the two sugars. In M2, both sucrose and lactose could be used by L90 taking into account the ability of L90 to use

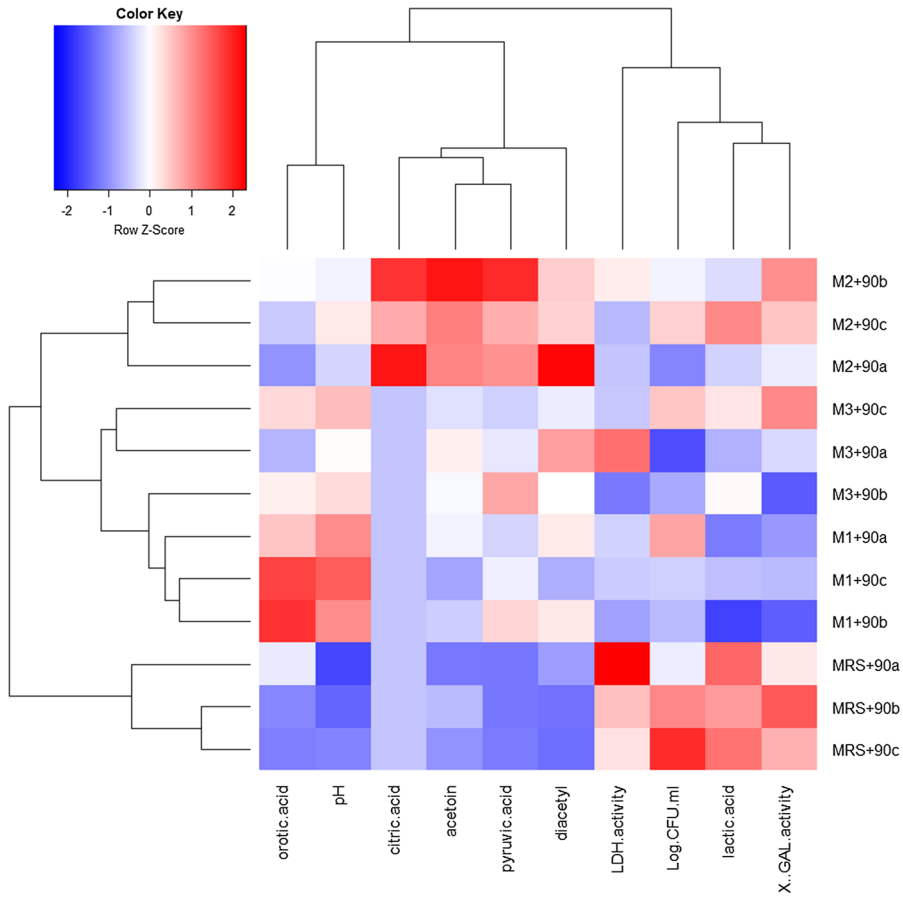


Fig. 6 Heat map of correlations between the enzyme activities levels of L90 and the levels organic acids, microbiological counts, diacetyl and acetoin in fermented milks

sucrose observed in M3, as well as the typical ability of lactobacilli to ferment lactose (Tamime et al. 2006), which has been documented for L90 (Peralta et al. 2014). The diminution of the peak with these overlapped sugars suggests that one or both were used as energy source by L90 in M2. Furthermore, the cheese whey permeate used as a source of lactose for the M2 usually contains salts and minerals (Ahmad et al. 2019) which could have been used by L90 as growth factors. The essential role of manganese, in trace quantities, for the growth and metabolic activities of lactic acid bacteria is widely known (Böhmer et al. 2013). On the other hand, the positive effect of copper on the production of biomass and diacetyl has been reported for lactic acid bacteria, as well (Kaneko et al. 1990). In addition, whey permeate also contains other compounds, such as amino acids, hippuric and orotic acids and vitamins (Frankowski et al. 2014), which could influence the growth and activity of L90. In the present work we evaluated the associated effect of all compounds present in the whey permeate without identify the individual influence of each one.

In LAB, the ability to metabolise different carbohydrates is usually regulated by the carbon catabolite repression system (Görke and Stülke 2008; Andreevskaya et al. 2016). In the present work, the ability of L90 to metabolise sucrose, observed in M3, was inhibited in the medium containing glucose (M1). In the same way, it has been shown a repression of the fructooligosaccharide utilization by *Lacticaseibacillus paracasei* 1195 due to the presence of glucose in the medium (Goh et al. 2007). This is also coincident with a more recent study by Zhong et al. (2018), who reported differences between the proteome of *Lactobacillus acidophilus* CICC22162 grown on MRS (with glucose) or on MRS modified replacing the glucose by

stachyose. They reported that the enzymatic activities α-galactosidase, mannose-6-phosphate isomerase, and invertase were higher in MRS-stachyose than in the MRS, suggesting that stachyose induced the expressions of these three proteins needed for its catabolism. On other hand, L90 presented different levels of LDH and β-GAL activities depending on which culture media it was grown. Similarly, Hickey et al. (1986) and Vasiljevic and Jelen (2001) also reported some variations between levels of β-GAL in lactobacilli strains depending on the carbohydrate present in the growth media.

Relating the levels of lactic and acetic acid with the pH values (Fig. 2C), a lower buffer capacity was observed in the optimized media (M1, M2, and M3) in comparison with MRS, which could be associated with the higher amount of components present in MRS (polypeptone, meat extract, sodium acetate, ammonium citrate, K_2HPO_4), which could maintain the pH. Many factors influence the ability of LAB to completely ferment sugars (Lu et al. 2001); in the present work, the differences of the pH values could be the reason why the consumption of glucose in MRS was complete, while it was partial in M1.

Yeast extract, and salts of magnesium and manganese are widely used for the formulation of culture media. The beneficial effect of yeast extract on the production of biomass and lactic acid, mainly by its high contribution of nitrogen compounds, has been reported for *Lacticaseibacillus rhamnosus* (Nancib et al. 2001), *Lactiplantibacillus plantarum* BL011 (Coghetto et al. 2016) and *Limosilactobacillus fermentum* (Gao et al. 2009), among others. In addition, the yeast extract contains B vitamins (thiamine, riboflavin, pyridoxine niacin, pantothenate, biotin, folic acid, and vitamin B12) that are cofactors for enzymes involved in essential reactions for cell functions (Gao et al. 2009; Ewe et al. 2010). In this sense, *Lacticaseibacillus paracasei* NERCB 0401 increased lactic acid production with the addition of vitamin B12 (Xu et al. 2008). The positive effect of magnesium and manganese on the growth of lactobacilli has been previously reported (Fitzpatrick et al. 2001; Groot et al. 2005). In fact, the incorporation of these minerals is frequent in formulations of culture media for LAB, since both are associated with the enzymatic functioning of numerous microorganisms (Fitzpatrick et al. 2001; Lavari et al. 2015). The Mn^{2+} ion is required in trace amounts by most bacterial species for its growth and survival since it also fulfils functions as an enzyme cofactor (Groot et al. 2005).

Although the levels reached by L90 at the end of incubation in milks were similar, interesting changes in lactose, lactic acid, pyruvic acid and pH values were found, which were correlated between them as well as with the β-GAL and LDH activities. The lowest and highest concentrations of lactose and lactic acid, respectively, which were noticed in FMmrs were associated with the highest β-GAL activity in CFEmrs. In contrast, the highest and lowest levels of lactose and lactic acid, respectively, presented in FMm1 were associated with the lowest β-GAL activity in CFEm1. Furthermore, the concentration of pyruvic and lactic acid were related with LDH activity, since the highest and lowest concentration of lactic acid and pyruvic acid in FMmrs, respectively, were associated with the highest LDH activity in CFEmrs. The lactic acid is the main product from glucose metabolism in homofermentative LAB formed via the glycolysis pathway and it occurs in the most fermented dairy products (Tamime et al. 2006). LDH and β-GAL are key intracellular enzymes that produce lactic acid via fermentation of lactose (Tamime et al. 2006).

Hippuric acid, naturally present in cow's milk, can be used by LAB and transformed into benzoic acid (Güzel-Seydim et al. 2000). In our work, L90 showed a high ability to ferment hippuric acid. In the same sense, the decrease of orotic acid could be due to the consumption of this acid as growth factor by LAB (González de Llano et al. 1996).

Pyruvate is a key metabolic intermediate in the central carbon metabolism in all organisms, including the LAB (Cocaign-Bousquet et al. 1996; Liu 2003). Thus, higher levels of this compound could be attributed to either an increased production or a decreased metabolism. In a previous study, L90 increased the levels of pyruvate in a model cheese (Peralta et al. 2016b), which was also observed in the present work during the milk fermentation. The glycolysis pathway is one of the main sources of pyruvate, however, there are other possible pathways, such as the metabolism of amino acids and citrate. Regarding their metabolism, different pathways are also possible, which led to the production of different compounds such as lactate, acetate, oxalacetate, and volatile compounds diacetyl and acetoin, between others (Le Bars and Yvon 2008; Zuljan et al. 2016).

Therefore, it is difficult to explain exactly the pathways that led to higher levels of pyruvate in the FMm2 in comparison with the other fermented milks.

On other hand, the notable decrease of citric acid in the fermented milks highlights the ability of L90 to metabolise it as an energy source, contrary to previous reports (Milesi et al. 2010; Peralta et al. 2017) which showed that L90 was not able to metabolise citrate when it was incubated in a cheese extract or used as an adjunct culture in soft cheese. These differences could be associated with higher concentrations of short peptides, amino acids, and galactose in the cheese extract and in the cheese compared to milk. However, the consumption of citrate was lower in FMm2 in comparison with the other fermented milks, which suggests that the enzymes involved in citrate metabolism could have been negatively affected by the growth of L90 in M2. Even though the level of citrate conversion in LAB is very low, it is an important feature of some mesophilic strains (Tamime et al. 2006). Citrate, normal component of milk, is transported into the cell by the citrate permease enzyme, transformed to oxaloacetate by citrate lyase and further degraded to pyruvate by a decarboxylase (Zuljan et al. 2016).

Diacetyl and acetoin are two important flavour compounds in many varieties of cheese, which can be produced from several metabolic pathways such as the metabolism of carbohydrates (lactose, citrate) and amino acids (Le Bars and Yvon 2008; Zuljan et al. 2016). The production of diacetyl and acetoin from citrate is common in LAB (García-Quintáns et al. 2008) and this could be the main pathway used by L90 in FMm1, FMm3 and FMmrs given the total consumption of citrate observed in these samples. However, the association of higher levels of these compounds and also higher levels of citrate in FMm2 suggest that other pathways were also used to produce diacetyl and acetoin in these samples. In particular, in previous works, we showed that L90 could produce these compounds from aspartate metabolism (Peralta et al. 2016a, b). Our results show the influence of the growth media on the metabolic activity of L90 in milk.

Conclusion

The three optimized media for the biomass production of the L90 strain allowed obtaining high levels (~ 9 log cfu mL^{-1}) that comply with industrial requirements. The growth of L90 in these media produced changes in its ability to metabolise lactose and citric acid, as well as in the enzymatic activities LDH and β-GAL, yielding differences in the pH values and concentrations of lactic and pyruvic acids in fermented milks. The ability of the strain to produce the key flavour compounds (diacetyl and acetoin) was maintained or improved by the growth in the optimized media in comparison with MRS. On the other hand, the highest levels of pyruvate, diacetyl and acetoin in FMm2, as well as the highest citrate concentrations, suggest that the production of these volatile compounds by L90 would not be related only to the citrate metabolism. These results suggest that the whey permeate added in M2 could promote other metabolic pathways in the L90 strain. However, more studies are needed in order to identify the individual influence of the compounds added to the media (lactose and other compounds present in whey permeate, compounds present in yeast extract) on the activity of L90.

Authors' contributions Laboratory work M.V.B. and G.H.P; Optimization design L.V.C.; Volatile compounds determination I.V.W.; Minerals determination R.S.; Writing original draft G.H.P, M.V.B. and C.V.B; Project administration, Conceptualization and Funding acquisition G.H.P; E.R.H.; C.V.B. All authors contributed to writing the manuscript.

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Data availability All data generated or analysed during this study are included in this article and its supplementary information files.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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