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ORIGINAL PAPER

Microcultures of lactic acid bacteria: characterization and selection of strains, optimization of nutrients and gallic acid concentration

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Abstract Eighteen lactic acid bacteria (LAB) strains, isolated from coffee pulp silages were characterized according to both growth and gallic acid (GA) consumption. Prussian blue method was adapted to 96-well microplates to quantify GA in LAB microcultures. Normalized data of growth and GA consumption were used to characterize strains into four phenotypes. A number of 5 LAB strains showed more than 60% of tolerance to GA at 2 g/l; whereas at 10 g/l GA growth inhibition was detected to a different extent depending on each strain, although GA consumption was observed in seven studied strains (>60%). *Lactobacillus plantarum* L-08 was selected for further studies based on its capacity to degrade GA at 10 g/l (97%). MRS broth and GA concentrations were varied to study the effect on growth of LAB. Cell density and growth rate were optimized by response surface methodology and kinetic analysis. Maximum growth was attained after 7.5 h of cultivation, with a dilution factor of 1–1/2 and a GA concentration between 0.625 and 2.5 g/l. Results indicated that the main factor affecting LAB growth was GA concentration. The main contribution of this study was to propose a novel adaptation of a methodology to characterize and select LAB strains with detoxifying potential of simple phenolics based on GA consumption and tolerance. In addition, the methodology presented in this study integrated the well-known RSM with an experimental design based on successive dilutions.

Keywords Lactic acid bacteria · Gallic acid · Microculture · Prussian blue method · Response surface methodology

Abbreviations

GA	Gallic acid
HPLC	High performance liquid chromatography
LAB	Lactic acid bacteria
MRS	Man Rogosa and Sharpe
OD	Optical density
PC	Phenolic compounds
RSM	Response surface methodology
X_1	Dilution factor
X_2	Gallic acid concentration
X_1	Coded dilution factor, $-\log_2(X_1)$
X_2	Coded GA, $-\log_2(X_2/10)$

Introduction

Phenolic compounds (PC) are secondary plant metabolites known for their ability to bind proteins, polysaccharides, fatty acids, or metal ions. Their presence in agro-industrial residues limits their use as animal feed mainly due to

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antinutritional and toxic effects [15, 22]. Some lactic acid bacteria (LAB) are often used as inoculants for silage or as probiotics for ruminants [7, 35] and can grow, tolerate or degrade PC. Several studies have found that LAB growth is affected by phenolics; the effects of PC were reported on growth and viability of *Oenococcus oeni* and *L. hilgardii* strains isolated from wine [3]. In the case of *O. ovni*, catechin and quercetin stimulated malolactic fermentation, but *p*-coumaric acid has an inhibitory effect [24]. In addition, several authors have reported PC consumption as mentioned by Cavin et al. [4]. They showed that *Lactobacillus* and *Pediococcus* strains, isolated from wine, were able to transform ferulic and *p*-coumaric acids producing volatile phenols (4-vynil derivatives and 4-ethyl derivatives). In a similar way, Alberto et al. [1] reported GA and catechin consumption in *L. hilgardii* 5w cultures; Osawa et al. [21] described the degradation of tannins by *Lactobacillus* strains from human feces and fermented foods and Vaquero et al. [33] isolated *L. plantarum* strains that produce tanin acil hydrolase (tanase, EC 3.1.1.20). A common pathway was reported for PC degradation in which tannic acid was hydrolyzed to gallic acid (GA) which was then decarboxylated producing pyrogallol (Fig. 1).

Coffee pulp is an important seasonal agro-industrial residue of the coffee industry. Silages of this material showed a decrease in total PC content attributed to the activity of LAB [8]. In addition, a *Lactobacillus* sp. strain ASR S1A, isolated from sheep excreta, produced an extracellular tannase under solid-state fermentation (SSF) using coffee husk as substrate [27].

The aim of the present study was to propose a practical methodology to characterize and select LAB strains, isolated from coffee pulp silages, according to their capacity of growth and utilization of GA. Moreover, the Prussian blue method was adapted to microplates. A selected strain was further characterized according to culture medium nutrients and GA concentration using a response surface methodology (RSM) supported by successive dilutions. Additionally, growth kinetics was evaluated using the Gompertz model.

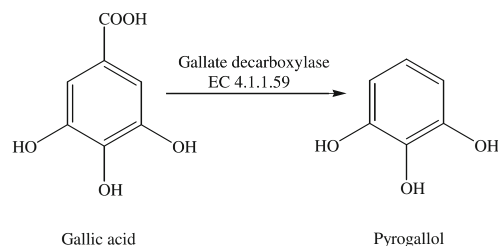

Fig. 1 Decarboxylation reaction of gallic acid (*GA*) to produce pyrogallol

Materials and methods

Microorganisms

Eighteen LAB strains belonging to the UAM-IRD collection (Institut de Recherche pour le Développement, France) were studied. Table 1 shows strains isolated from coffee pulp silage (obtained from Xalapa, Veracruz, México) and were primarily identified by the MIDI system (Microbial Identification System; Microbial ID, Newark, Delaware, USA), based on analysis of bacterial fatty acid's concentration compared with an internal standard [9].

Maintenance and conservation of LAB strains

Microorganisms were maintained in standard MRS (Man Rogosa and Sharpe) broth (Difco TM, Becton, Dickinson and Company, Sparks, USA) and were stored at 4°C. LAB strains were re-cultured each month and preserved at −30°C in MRS broth supplemented with sterile glycerol 30% v/v [11].

Culture media

A modified medium of MRS, named in this study mMRS, was prepared with the original components of MRS broth [5]. Glucose concentration was modified according to experiments, as indicated in Table 2. Initial pH was adjusted to 6.8. Glucose was purchased from J.T. Baker

Table 1 LAB strains (UAM-IRD collection) studied

Code	Strain name
L-03	*Lactobacillus buchneri*
L-04	*Lactobacillus plantarum*
L-06	*Pediococcus pentosaceus*
L-08	*Lactobacillus plantarum*
L-11	*Lactobacillus plantarum*
L-12	*Lactobacillus plantarum*
L-16	*Pediococcus pentosaceus*
L-18	*Pediococcus pentosaceus*
L-19	*Lactobacillus plantarum*
L-20	*Pediococcus pentosaceus*
Nat-1	*Weissella confusa*
Nat-5	*Weissella confusa*
Nat-7	*Lactobacillus pentosus*
Nat-16	NI
Nat-21	*Weissella confusa*
Nat-38	*Lactobacillus plantarum*
IG-1	*Lactobacillus hilgardii*
AMR1	NI

NI not identified

Table 2 Culture media composition at different stages

	Inocula preparation[a]	Screening studies[b]	Response surface studies[b]
Dilution factor	1	2	1, 1/2, 1/4, 1/8, 1/16, 1/32
Glucose (g/l)	20	1.5	1.5
Gallic acid (g/l)	0.3	2, 10, 20	10, 5, 2.5, 1.25, 0.625, 0.3125

[a] Standard MRS broth

[b] Modified MRS broth (mMRS)

Inc. (Philipsburg, NJ, USA). Culture media were sterilized by autoclaving at 121°C for 15 min.

Addition of gallic acid

Gallic acid (Sigma Aldrich Inc., St. Louis, USA) was used as a simple phenolic added to cultures. This compound was dissolved in water and the pH was adjusted to 6.8 with NaOH 1 N. GA solutions were filtered through a sterile membrane filter (0.22 µm pore size, Millipore, Bedford, MA, USA) and added after mMRS broth sterilization.

Inocula preparation

Inocula were prepared in test tubes (15 ml) containing 10 ml of mMRS broth to which 0.3 g/l GA was added. Culture bacteria were inoculated at 5% (v/v) with cells cultivated for 24 h at 37°C in darkness and without stirring. Cells were washed with NaCl (0.9% w/v) and centrifuged (5,000 rpm, 10 min). Inocula were standardized to an absorbance of 0.05 optical density units (ODU) corresponding to 0.5 McFarland turbidity scale, reported as 1.5×10^{10} colony forming units (CFU)/ml [14].

Adaptation of the Prussian blue method in microplates

The Prussian blue method was used to determine GA concentration in 96-well microplates but some modifications were carried out in order to avoid interferences caused by proteins of MRS broth. Different protein precipitating agents were tested: Trichloracetic acid (TCA, 10%), $CaCl_2$ (300 mM), $ZnSO_4$ (300 mM), $BaCl_3$ (300 mM), and methanol (reactive grade), which were added to mMRS broth in Eppendorf tubes in 2:1 (v/v) proportion in all the cases. Additionally, concentration and volume of Prussian blue components were adjusted in order to be used in microplates. The final protocol is presented in analytical techniques.

Screening of strains

Experiments were carried out in 96-well microplates (Costar® 3799, NY, USA) using mMRS broth diluted to 50% (w/v) in water containing glucose (1.5 g/l). GA was added at 2, 10 and 20 g/l. Culture medium was distributed in each well (160 µl) inoculated with 30 µl of standardized inoculum. Microplates were incubated at 37°C in darkness and without stirring. Optical density (OD) was measured after 20 h of cultivation. Adequate sterility control was prepared adding 30 µl of NaCl 0.9% (v/v) instead of inoculum; no contamination was observed after incubation. All experiments were performed in duplicate.

Microplates were prepared separately under identical conditions and GA quantification was carried out at 0 and 44 h of culture. Cell growth and GA consumption were criteria for characterization and selection of LAB strains. GA consumption and OD measurements were normalized with respect to the maximum result obtained and plotted in a Cartesian coordinate system [26]. Thus, LAB strains were characterized into four quadrants corresponding to four different phenotypes.

Effect of nutrients and GA concentrations using RSM

Cultivation of the selected strain (*L. plantarum* L-08) was performed in mMRS at different broth dilutions and different GA concentrations. Cultures were carried out in 96-well microplates (Costar® 3799, NY, USA). mMRS broth was diluted according to the following six dilution factors (X_1): 1, 1/2, 1/4, 1/8, 1/16 and 1/32; whereas six GA concentrations (X_2) were tested: 10, 5, 2.5, 1.25, 0.625 and 0.3125 g/l. All conditions were prepared using successive dilutions [2, 13]. GA solutions were prepared separately and were added to diluted mMRS broth in order to carry out 36 experiments which covered the entire range of factor combinations examined (Table 3).

Each well was prepared with 150 µl mMRS broth plus 50 µl GA solution, followed by inoculation with 30 µl of previously standardized LAB. Microcultures were incubated in a microplate reader (Ultra Microplate Reader ELX808 IU, Biotek Instruments) at 37°C. Growth was measured at 0, 2, 4, 5, 6 and 7.5 h after stirring of microplates. All assays were performed in duplicate and adequate sterility and growth controls without GA were included.

The effect of mMRS dilution and GA concentration upon growth of *L. plantarum* L-08 strain was analyzed by using RSM. Logarithmic transformations were applied to the mMRS broth dilution factor (X_1) and GA concentration (X_2) as follows: $X_1 = -\log_2(X_1)$; $X_2 = -\log_2(X_2/10)$, as indicated in Table 3. The $\log_2$ is widely used in microdilution studies like minimum inhibitory concentrations (MICs). In this way, data showed a homogeneous

Table 3 Experimental design of 36 combinations showing 2 variables with real values and coded values (transformed logarithmically)

Run no.	Dilution factor (X_1)		GA (g/l) (X_2)	Coded dilution[a] (X_1)	Coded GA[b] (X_2)
1	1=	1	0.3125	0	5
2	1/2=	0.5	0.3125	1	5
3	1/4=	0.25	0.3125	2	5
4	1/8=	0.125	0.3125	3	5
5	1/16=	0.0625	0.3125	4	5
6	1/32=	0.03125	0.3125	5	5
7	1=	1	0.625	0	4
8	1/2=	0.5	0.625	1	4
9	1/4=	0.25	0.625	2	4
10	1/8=	0.125	0.625	3	4
11	1/16=	0.0625	0.625	4	4
12	1/32=	0.03125	0.625	5	4
13	1=	1	1.25	0	3
14	1/2=	0.5	1.25	1	3
15	1/4=	0.25	1.25	2	3
16	1/8=	0.125	1.25	3	3
17	1/16=	0.0625	1.25	4	3
18	1/32=	0.03125	1.25	5	3
19	1=	1	2.5	0	2
20	1/2=	0.5	2.5	1	2
21	1/4=	0.25	2.5	2	2
22	1/8=	0.125	2.5	3	2
23	1/16=	0.0625	2.5	4	2
24	1/32=	0.03125	2.5	5	2
25	1=	1	5	0	1
26	1/2=	0.5	5	1	1
27	1/4=	0.25	5	2	1
28	1/8=	0.125	5	3	1
29	1/16=	0.0625	5	4	1
30	1/32=	0.03125	5	5	1
31	1=	1	10	0	0
32	1/2=	0.5	10	1	0
33	1/4=	0.25	10	2	0
34	1/8=	0.125	10	3	0
35	1/16=	0.0625	10	4	0
36	1/32=	0.03125	10	5	0

[a] $X_1 = -\log_2(X_1)$

[b] $X_2 = -\log_2(X_2/10)$

distribution in the range of values studied [2, 13]. Thus, the transformed variables varied from 0 to 5 (Table 3).

The effect of transformed factors (X_1 and X_2) on cell growth at different cultivation times (0, 2, 4, 5, 6 and 7.5 h) was described by using a second order polynomial model:

$$OD = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

where OD is the predicted response for cell growth, β_0 is an independent constant; β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic regression coefficients; andβ_{12} is the interaction coefficient.

Regressions and analyses of variance (ANOVA) were evaluated for significance (F test, $P < 0.05$) and the determination coefficients (R^2 values) were also estimated. Significance of the regression coefficients were tested by a t test [17].

LAB kinetics

Time course of OD_{595} in the microplates of *L. plantarum* L-08, for the 36 conditions mentioned above, was described using the Gompertz model, the integral form of which is given by Eq. 2:

$$X = X_{max} e^{\left(-b e^{(-kt)}\right)} \quad (2)$$

where X is the adjusted OD, X_{max} is the maximal OD, k is a growth constant with a specific significance and t is time. The initial condition for OD is $X_0 = X_{max} e^{(-b)}$, where b is a defined positive number associated to initial culture conditions [28]. Growth rates (k value) and R^2 values were calculated for each culture condition and were represented in a 3D surface plot.

Furthermore, the μ values of growth kinetics were calculated for the same 36 conditions in the exponential growth phase (μ = Ln (ΔOD_{595})/Δt, where t is time).

Statistical analysis

Statistical analysis of screening data of LAB and kinetic analysis were performed by *Microsoft Excel* (Microsoft Corp., Redmond, WA, USA). SigmaPlot® version 8.02 for Windows® (Jandel Scientific, San Rafael, CA, USA) was used to carry out the regression analysis and to generate the 3D plots.

Analytical techniques

Optical density

Biomass was estimated by optical density of microcultures measured at 595 nm using an *ELx808 IU* Ultra microplate reader and the *KC4* software version 3.2, BioTek Instruments, Winooski, VT, USA [12]. Inoculum standardization was carried out with OD readings at 600 nm (Lambda 25 UV/VIS Spectrometer, Perkin-Elmer).

Prussian blue method in microplates

Prussian blue method was used to determine GA concentration [30] in 96-well microplates (Costar® 3799, NY, USA). The protocol is as follows: Add 5 µl sample, 50 µl $FeCl_3$ (20 mM, JT Baker Inc., Philipsburg, NJ, USA) and 50 µl $K_3Fe(CN)_6$ (16 mM, JT Baker Inc., Philipsburg, NJ, USA). After 10 min 150 µl of a stabilizer was added.

Reaction principle is based on oxidation–reduction reactions wherein the phenolate ion is oxidized while $Fe(CN)_6^{3-}$ ion is reduced, thereby forming a blue colored complex $Fe_4[Fe(CN)_6]_3$ named Prussian blue, quantified by spectrophotometry and intensity of color formed that is directly proportional to PC concentration [23]. Arabic gum was used as stabilizer according to Graham et al. [10].

Samples were treated with methanol in order to eliminate interferences of proteins in mMRS broth [29]. This procedure consisted of adding 150 µl of culture broth and 300 µl of methanol in Eppendorf tubes which were centrifuged (10,000 rpm, 5 min). A supernatant aliquot was taken for the Prussian blue reaction. Then absorbance was read in duplicates at 595 nm using a microplate reader (Ultra Microplate Reader ELX808 IU, Biotek Instruments). Samples were prepared in the range of validity of the technique and standard plots were carried out in triplicates with GA as standard.

Results and discussion

Adaptation of the Prussian blue method in microplates

Quantification of PC uses methods such as HPLC with UV detection and GC/MS [32], as well as several colorimetric methods (Folin-Ciocalteu, Folin-Denis, and Prussian blue) and other techniques based on protein precipitation [18]. Prussian blue method is a simple technique showing short response times (<15 min); however, there are interferences caused by protein precipitation due to the presence of $FeCl_3$, which represents an obstacle for the use of this method in MRS broth cultures. For this purpose, different precipitating agents were tested: Trichloracetic acid (TCA), $CaCl_2$, $ZnSO_4$, and $BaCl_3$, although interferences were observed in all the cases. Addition of methanol to samples in proportion 2:1 (methanol:mMRS broth, v/v) enhanced protein precipitation and eliminated interferences. Standard plots were performed with GA ($y = 2.7366x + 0.0154$, $R^2 = 0.9974$) and pyrogallol ($y = 4.8956x + 0.074$, $R^2 = 0.9921$); a linear plot was obtained for concentrations up to 0.5 g/l for each compound. It should be noted that chemical reaction in this method appears after aromatic ring breakage, without distinguishing between compounds with different substituents, for example, GA and pyrogallol (Fig. 1).

It is important to emphasize that adaptation of Prussian blue method to microplates represents a potential of application due to simplicity and accuracy of the technical procedure. It is worth mentioning, that this method has not previously been used for the study of degradation of simple PC by LAB.

Screening of LAB

Strains were cultivated at 2, 10 and 20 g/l of GA. Normalized growth and GA consumption of 2 and 10 g/l of GA are shown in Fig. 2a, b. No growth or GA consumption was observed at 20 g/l for all LAB strains tested. Each quadrant of the Cartesian plot represents a different phenotype. The two upper quadrants (I and II, Fig. 2a, b) identify LAB strains with a higher growth in presence of different GA concentrations in comparison with the lower quadrants (III and IV, Fig. 2a, b). Quadrants on the right side (I and IV) identify LAB strains with increased

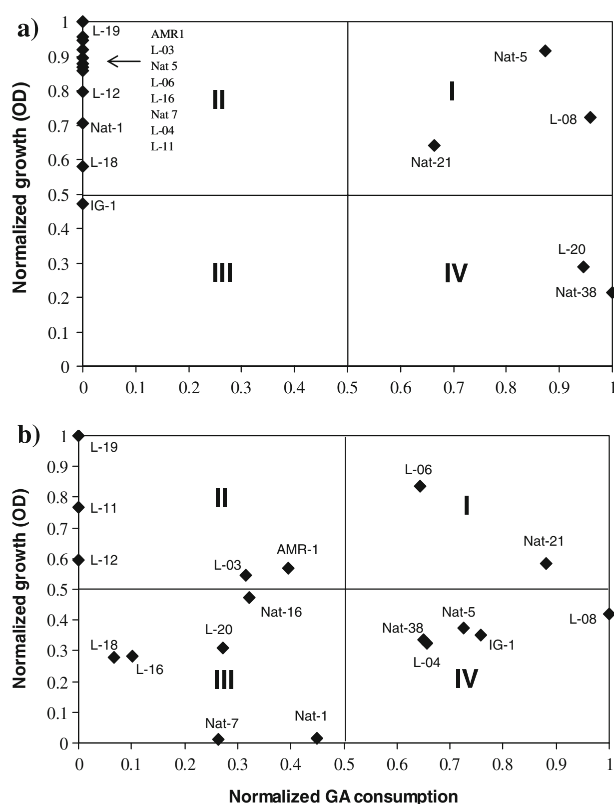

Fig. 2 Cartesian quadrant plots of normalized growth data and GA consumption of 18 LAB strains studied at two different GA concentrations: **a** 2 g/l and **b** 10 g/l. Incubation time was 44 h, GA consumption and OD measurements were normalized with respect to the maximum result obtained

capacity of GA consumption in comparison with bacteria which are located in the left side quadrants (II and III).

In Fig. 2a, a total of 13 out of 18 strains did not utilize GA, whereas, 3 out of 18 (IG-1, L-20, Nat-38) presented less than 50% of the maximum growth at low GA concentration (2 g/l). Growth inhibition did not take place in 13 studied strains. GA was, however, used by 5 strains (> 60%). In addition, higher GA concentration (10 g/l) caused a wider dispersion of the observed phenotypes (Fig. 2b) in comparison with the lower GA concentration (Fig. 2a). At 10 g/l GA, 11 strains presented less than 50% of the maximum growth and 7 strains degraded more than 60% of the GA originally present. LAB were affected by GA concentration in media; at low GA concentration, inhibition was not observed, however, GA was not metabolized either; whereas, at 10 g/l growth inhibition was higher and GA consumption was stimulated for 7 strains. The greater number of strains in lower quadrants suggests that higher GA concentration presents a greater inhibition (Fig. 2b).

Lactobacillus plantarum L-08 strain showed a high GA consumption (>97% at 44 h) at initial concentration of 2 and 10 g/l GA. These concentrations were higher than those studied by Alberto et al. [1], who tested GA concentrations of 0.05, 0.1 and 0.2 g/l and the consumptions achieved were 60, 51 and 30%, respectively, in MRS media. *Lactobacillus plantarum* L-08 was selected because of its potential applications.

It is worth mentioning, that during inoculum preparation, LAB cultures contained small amounts of easily assimilable sugar (initial glucose = 1.5 g/l). This condition probably stimulated the use of GA in some strains. A similar strategy was used by Alberto et al. [1], where a *L. hilgardii* strain 5w was previously cultivated in MRS media in the presence of GA (0.1 g/l) or catechin (0.2 g/l), and increasing values of μ_{max}, cell density and glucose consumption rate were reported. Our results of GA consumption are in agreement with this approach.

In this study, an inhibitory effect was observed, in agreement with previous reports. Campos et al. [3] found that hydroxybenzoics acids (*p*-hydroxybenzoic, protocatechuic, gallic, vanillic, and syringic acids) and hydroxycinnamics acids (*p*-coumaric, caffeic and ferulic acids) affected *O. oeni* and *L. hilgardii* growth. In both strains, the *p*-coumaric acid showed the strongest inhibitory effect on growth. In the case of *O. oeni* growth, hydroxycinnamic acids exhibited a higher inhibitory effect than hydroxybenzoic acids. Similarly, for *L. collinoides* and *L. brevis* strains, hydroxycinamics acids inhibited growth in presence of GA at 0.5 and 1 g/l [31]. Moreover, Alberto et al. [1] reported an inhibitory effect on *L. hilgardii* 5w growth (10 and 26% at initial GA 1 and 3 g/l, respectively). The effect of PC depended on the bacterial strain, in addition to the specific PC tested and its concentration. The inhibitory effect could be explained by PC adsorption on cell walls [34], accumulation of dissociated or undissociated phenolic acids in the cytoplasm [24], and damage to cell membrane [16].

The methodology presented in this work is a practical tool and has a significant potential for the screening of strains, showing either PC tolerance or ability to consume such compounds. It could be useful in the screening of strains as is the case of ruminal bacteria [19, 20] or genetically modified LAB. In this work, the GA concentrations tested were 50–200 times higher than that reported by Alberto et al. [1].

Effect of nutrients and GA concentration using RSM

As mentioned above, *L. plantarum* L-08 strain was selected because of its capacity of GA consumption and acceptable growth. RSM has been widely used in microbial optimization studies, for example, in LAB cultures it was recently applied to obtain products of industrial interest such as bacteriocins [6] or biosurfactants [25]. In addition the central composite design is extensively used, with factor values usually changing in the same order of magnitude. On the other hand, this paper proposed an experimental

design based on successive dilutions (1:2) as indicated in "Material and methods", with the advantage that variation of the factors is wider (32 times) in comparison with the lower and higher values of factors tested, while the benefits of visualization of the RSM are maintained. An appropriate logarithmic base should be used when the dilution factor is changed in successive stages, $\log_2$ for 1/2, $\log_{10}$ for 1/10, etc. As the dilution factor is decreased wider is the variation of the factor values.

The effect of transformed mMRS dilution factor (X_1, coded dilution) and GA concentration (X_2, coded GA) upon growth of *L. plantarum* L-08 strain was studied by using RSM. The experimental data for each sampling time (0, 2, 4, 5, 6 and 7.5 h) were analyzed using the Fisher's statistical test for ANOVA and a summary of results is shown in Table 4; the RSM plots of 2, 4, 5 and 7.5 h of cultivation, are shown in Fig. 3. Higher goodness of fit according to the R^2, adjusted R^2 and standard error were observed for 4, 5, 6 and 7.5 h of cultivation, while very low P values were observed ($P < 0.0001$) when culture time increased. These data confirmed that the models were highly significant.

The regression coefficients for the different cultivation times are shown in Table 5, their significance (P value <0.05) was determined by Student's t test and P values. In this study, the effect on growth of the dilution (X_1), GA concentration (X_2), their interaction (X_1X_2) and the quadratic GA (X_2^2) were significant starting at 4 h of cultivation; quadratic dilution (X_1^2) was not significant in any of the cases. Partial regression coefficients suggest that the GA concentration is the most important factor affecting cell growth. It could be explained by the inhibitory effect of GA, as mentioned earlier.

The greatest OD values were obtained at 7.5 h of culture (Fig. 3). Experimental and predicted data of the model for this culture time are shown in Table 6. At this time, the ANOVA of the quadratic regression (Table 5) indicated that the model was significant, as the F value was 99.4. The value of R^2 (0.88) means that the model explains 88% of the variability in the data. The term X_1^2 was neglected on the basis of P value (Table 5). The reduced model could therefore be written as follows:

Table 4 Analysis of variance (ANOVA) for the second order models at different times of cultivation

Values	Time (h)					
	0	2	4	5	6	7.5
Model F value	6.2	15.9	86.7	89	125.1	99.4
P value	9E-05	3E-10	1E-27	6E-28	3E-32	2E-29
R^2	0.320	0.5459	0.868	0.8709	0.905	0.883
Adjusted R^2	0.269	0.5115	0.858	0.8611	0.897	0.874
Standard error	0.010	0.0527	0.075	0.1108	0.107	0.117

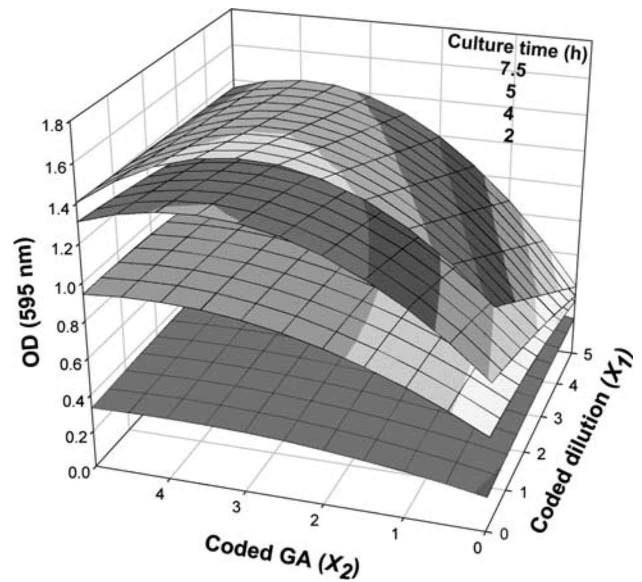

Fig. 3 Response surface plot showing the interactive effect between coded GA concentration and coded dilution of mMRS upon growth of *Lactobacillus plantarum* L-08 at 2, 4, 5 and 7.5 h of cultivation

Table 5 Coefficients of regression at 4, 5, 6 and 7.5 h of cultivation

Time (h)	Model term	Coefficients	Standard error	t value	P value
4	X_0*	0.5073	0.0322	15.7407	<0.0001
	X_1	−0.0096	0.0198	−0.4828	0.6308
	X_2*	0.2141	0.0198	10.7940	<0.0001
	X^2_1	−0.0055	0.0035	−1.5500	0.1259
	X_2^2*	−0.0255	0.0035	−7.2265	<0.0001
	X_1X_2	0.0037	0.0030	1.2269	0.2242
5	X_0*	0.7855	0.0479	16.4001	<0.0001
	X_1*	−0.0681	0.0295	−2.3113	0.0239
	X_2*	0.3571	0.0295	12.1110	<0.0001
	X^2_1	−0.0036	0.0052	−0.6886	0.4935
	X_2^2*	−0.0504	0.0052	−9.6163	<0.0001
	X_1X_2	0.0089	0.0045	1.9941	0.0503
6	X_0*	0.9925	0.0462	21.4982	<0.0001
	X_1*	−0.1156	0.0284	−4.0677	0.0001
	X_2*	0.4037	0.0284	14.2083	<0.0001
	X^2_1	−0.0019	0.0050	−0.3739	0.7097
	X_2^2*	−0.0630	0.0050	−12.4871	<0.0001
	X_1X_2*	0.0188	0.0043	4.3473	<0.0001
7.5	X_0*	1.1586	0.0507	22.8704	<0.0001
	X_1*	−0.1486	0.0312	−4.7669	<0.0001
	X_2*	0.3868	0.0312	12.4034	<0.0001
	X^2_1	−0.0001	0.0055	−0.0247	0.9804
	X_2^2*	−0.0674	0.0055	−12.1776	<0.0001
	X_1X_2*	0.0278	0.0047	5.8742	<0.0001

* Significant ($P < 0.05$)

Table 6 Experimental and predicted OD values of *L. plantarum* L-08 at 7.5 h of cultivation using 36 conditions carried out in duplicates

Run no.	X_1[a]	X_2[b]	OD (595 nm)		Run no.	X_1[a]	X_2[b]	OD (595 nm)	
			Observed	Predicted				Observed	Predicted
1	0	5	1.57	1.41	37	0	2	1.69	1.66
2	0	5	1.44	1.41	38	0	2	1.53	1.66
3	1	5	1.51	1.40	39	1	2	1.65	1.57
4	1	5	1.46	1.40	40	1	2	1.51	1.57
5	2	5	1.45	1.39	41	2	2	1.54	1.48
6	2	5	1.50	1.39	42	2	2	1.48	1.48
7	3	5	1.36	1.38	43	3	2	1.45	1.38
8	3	5	1.42	1.38	44	3	2	1.49	1.38
9	4	5	1.37	1.37	45	4	2	1.37	1.29
10	4	5	1.42	1.37	46	4	2	1.44	1.29
11	5	5	1.35	1.36	47	5	2	1.11	1.19
12	5	5	1.36	1.36	48	5	2	1.36	1.19
13	0	4	1.60	1.63	49	0	1	1.65	1.48
14	0	4	1.45	1.63	50	0	1	1.57	1.48
15	1	4	1.55	1.59	51	1	1	1.38	1.36
16	1	4	1.49	1.59	52	1	1	1.53	1.36
17	2	4	1.51	1.55	53	2	1	1.27	1.24
18	2	4	1.48	1.55	54	2	1	1.45	1.24
19	3	4	1.43	1.51	55	3	1	1.00	1.11
20	3	4	1.47	1.51	56	3	1	1.30	1.11
21	4	4	1.38	1.48	57	4	1	0.77	0.99
22	4	4	1.42	1.48	58	4	1	1.20	0.99
23	5	4	1.29	1.44	59	5	1	0.69	0.87
24	5	4	1.38	1.44	60	5	1	1.05	0.87
25	0	3	1.68	1.71	61	0	0	1.08	1.16
26	0	3	1.49	1.71	62	0	0	1.29	1.16
27	1	3	1.66	1.65	63	1	0	0.86	1.01
28	1	3	1.52	1.65	64	1	0	1.15	1.01
29	2	3	1.57	1.58	65	2	0	0.56	0.86
30	2	3	1.53	1.58	66	2	0	0.82	0.86
31	3	3	1.51	1.52	67	3	0	0.48	0.71
32	3	3	1.51	1.52	68	3	0	0.70	0.71
33	4	3	1.44	1.45	69	4	0	0.45	0.56
34	4	3	1.47	1.45	70	4	0	0.66	0.56
35	5	3	1.42	1.38	71	5	0	0.41	0.41
36	5	3	1.43	1.38	72	5	0	0.42	0.41

[a] Coded dilution

[b] Coded GA (Gallic acid); both are defined in Table 3

$$OD = 1.1586 - 0.1486X_1 + 0.3868X_2 - 0.0674X_2^2 + 0.0278X_1X_2$$

In the 3D response surface plot (Fig. 3), the visualization of the predicted growth showed that higher growth was attained in the zone comprising a dilution factor of 1 to 1/2 ($0 \leq X_1 \leq 1$) and a GA concentration of 2.5 to 0.625 g/l ($2 \leq X_2 \leq 4$).

The methodology presented in this study integrated the well-known RSM with an experimental design based on successive dilutions widely used in microbiological studies. This methodology allowed the optimization of growth as a function of dilution of MRS broth and GA concentration. This study was carried out for the *L. plantarum* L-08 strain; however, this technique could be applied to other strains.

Kinetics of *L. plantarum* L-08 strain

Analysis of growth kinetics of the selected strain, *L. plantarum* L-08, was carried out in microplates. All possible combinations of mMRS dilutions and GA concentrations were examined. The calculated growth rates

(k values) of the Gompertz model are presented in a 3D mesh plot (Fig. 4). The greatest k values were found in the dilution factors of 1 to 1/8 ($0 \leq X_1 \leq 3$) and a GA concentration of 1.25–0.3125 g/l ($3 \leq X_2 \leq 5$). Higher growth rates were found at low GA concentrations and diluted medium, in agreement with the higher OD observed (Fig. 3). It is noticeable that an increase in the growth rate (k) was observed in all the dilutions with high GA concentrations corresponding to the zone delimited by $0 \leq X_1 \leq 5$ and $0 \leq X_2 \leq 1$ (Fig. 4); nevertheless, cell growth was 75% lower than the maximum growth observed (Fig. 3). This could be explained by the adaptation of the LAB strain to GA during inoculum preparation.

The goodness of fit of the Gompertz model was verified by determination coefficients (R^2) represented in a 3D mesh plot (Fig. 4). The higher R^2 were found in the zone between $0 \leq X_1 \leq 5$ and $3 \leq X_2 \leq 5$, which is tested at all dilutions and a GA concentration between 1.25 and 0.3125 g/l. The behavior of k and R^2 values could be explained by low GA concentration and dilution of nutrients.

In this study, μ values of growth kinetics were calculated for each condition; a linear correlation was found between the k and μ values ($k = 1.12\mu$, $R^2 = 0.8$, then $k > \mu$). It occurred when k was higher than 0.4 h^{-1} (Fig. 4), for all the dilutions ($0 \leq X_1 \leq 5$) and at low GA concentrations tested ($2 \leq X_2 \leq 5$). The k values of Gompertz model can be interpreted as a specific growth parameter, but it should not be mistaken with the μ parameter of the classical exponential model.

Alberto et al. [1] reported a $\mu = 0.45$ h^{-1} for *L. hilgardii* 5w cultivated in MRS medium added with GA (0.1 g/l). In the present study, the conditions of higher k (0.63 h^{-1}) give a calculated μ of 0.58 h^{-1}; it is 20% higher than the value reported by Alberto et al. [1], despite higher concentrations of GA used in this study of *L. plantarum* L-08.

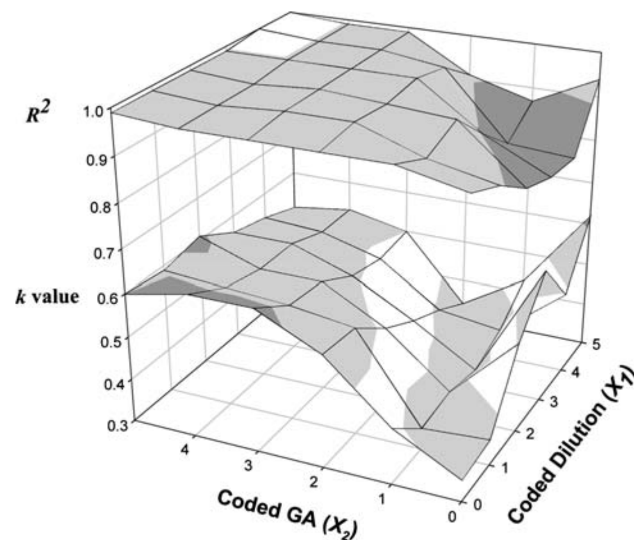

Fig. 4 Three-dimensional mesh plot showing the interactive effect of k and R^2 values calculated with the integral form of the Gompertz model and the coded dilution of mMRS (X_1) and the GA concentration (X_2) during the cultivation of *L. plantarum* L-08 at 7.5 h

Some typical microbial *S*-shape curves, described by the Gompertz model, are shown in Fig. 5, the growth profiles are influenced by different growing conditions. Analysis of these results can be carried out in combination with those on RSM plots (Fig. 3). Maximum growth was found in a zone between $0 \leq X_1 \leq 1$ and $2 \leq X_2 \leq 4$. In the same conditions higher growth rates and higher R^2 were found (Fig. 4). Experimental error seemed to be higher as the GA concentration increased resulting in a low goodness of fit determined by R^2. RSM and kinetic analysis could be useful to define culture conditions in order to attain high OD, higher growth rate and low experimental error for further studies.

In conclusion, a methodology was proposed in order to characterize and select LAB strains isolated from an agro-industrial residue, namely coffee pulp. The Prussian blue method was adapted to determine GA concentration in 96-well microplates. It is a simple and practical technique for screening of LAB. Normalized values of growth and GA consumption for the LAB strains were used as criteria to describe four phenotypes of LAB. In addition, the effect of dilution factor of mMRS and GA concentration upon growth of a selected strain (*L. plantarum* L-08) was examined. An experimental design based on successive dilutions and analyzed by RSM was used, while growth kinetics were examined using the Gompertz model. Best results for maximum cell density and growth rate were attained in a zone comprising a dilution factor of mMRS broth of 1–1/2 and a GA concentration of 0.625–2.5 g/l. Regression results suggested that the GA concentration was the most important factor affecting cell growth, which could be explained by the inhibitory effect.

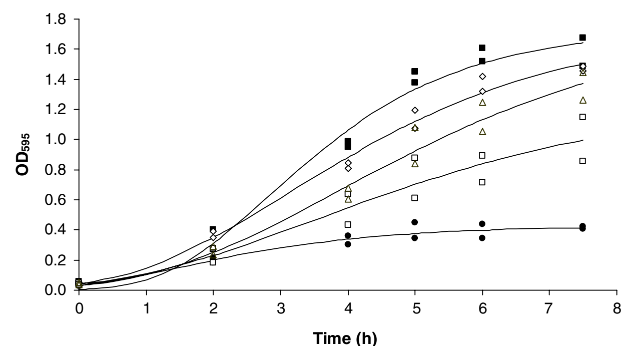

Fig. 5 Typical adjusted growth curves (*continuous line*) using the integrated form of the Gompertz model, at different conditions of coded dilution factor (X_1) and coded GA concentration (X_2). Experimental data of the following conditions of X_1 and X_2, respectively: (*filled square*) 0, 3; (*diamond*) 3, 2; (*triangle*) 2, 1; (*open square*) 1, 0; (*filled circle*) 5, 0

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