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Growth kinetic model, antioxidant and hypoglycemic effects at different temperatures of potential probiotic *Lactobacillus* spp

Modelado de las cinéticas de crecimiento, actividad antioxidante e hipoglucemiante a diferentes temperaturas de *Lactobacillus* spp. potencialmente probióticos

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Abstract

In this research, the growth, antioxidant and hypoglycemic activity of *Lactobacillus plantarum*, *L. pentosus* and *L. acidipiscis* isolated were evaluated at 32, 37 and 42 ºC. The three potential probiotic strains were isolated from a salty-ripened Mexican tropical cheese (double cream Chiapas cheese). The Unified-Gompertz equation was used to model the bacterial growth of the strains (R > 0.987). Similar biomass production was obtained at the end of the experiment (24 h) at 32 °C and 37 ºC. The maximum absolute growth rate was 0.526 h^{-1} for *L. acidipiscis* at 37 ºC. The antioxidant activity resulted maximum on DPPH at 32 ºC and 14 h in *L. pentosus* (7402.62 ± 318.52 μM Trolox/mL) and on ABTS at 32 ºC and 24 h in *L. acidipiscis* (1612.94 ± 56.71 μM Trolox/mL).The inhibition of α-amylase (%) and α-glucosidase (%) resulted maximum at 37 °C and 24 h for *L. acidipiscis* (97.084 ± 0.752 and 99.756 ± 0.104, respectively). These results suggest the relevance of the temperature and the growth phase when select potential probiotic strains on the viability and functional properties of the strains used in this work. The *in vivo* effect of the technological processes on the functionality of the strains are currently under research in our group.

Keywords: Halotolerant, U-Gompertz model, Stress, bioactiv, mesophiles or cheese.

Resumen

En esta investigación se analizó el crecimiento, actividad antioxidante e hipoglucemiante a 32, 37 y 42 °C de *Lactobacillus plantarum*, *L. pentosus* and *L. acidipiscis*. Las tres cepas potencialmente probióticas fueron aisladas de un queso Mexicano tropical salado madurado (queso doble crema de Chiapas). La ecuación Unificada de Gompertz se usó para modelar el crecimiento bacteriano de las cepas (R> 0.987). Se obtuvo una producción de biomasa similar al final del experimento (24 h) a 32 y 37 ºC. La tasa de crecimiento absoluta máxima fue de 0.526 h^{-1} para *L. acidipiscis* a 37 °C. La actividad antioxidante resultó máxima sobre DPPH a 32 °C y 14 h en *L. pentosus* (7402.62 ± 318.52 μM Trolox/mL); y sobre ABTS a 32 °C y 24 h en *L. acidipiscis* (1612.94 ± 56.71 μM Trolox/mL). La inhibición de la α-amilasa (%) y la α-glucosidasa (%) resultó máxima a 37 °C y 24 h para *L. acidipiscis* (97.084 ± 0.752 y 99.756 ± 0.104). Estos resultados sugieren la relevancia de las temperaturas y la fase de crecimiento bacterina en la selección de cepas potencialmente probióticas sobre su viabilidad y propiedades funcionales. El efecto *in vivo* de estos procesos y sobre la funcionalidad de las cepas está actualmente siendo investigada por nuestro grupo de trabajo.

Palabras clave: halotolerantes, Modelo de U-Gompertz, estrés, bioactivo, mesófilos o queso.

1 Introduction

Lactobacillus spp. play a major role in the lactic fermentation of traditional and industrial foodstuffs (dairy products, sourdough, meat and vegetables) because of their ability to produce organic acids (mainly lactic acid) which reduces the pH, increases the shelf life and give unique sensorial characteristics (Venegas-Ortega *et al*., 2019). Moreover, they are natural inhabitants of a healthy microbiota (Kumar *et al*., 2015).

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Some *Lactobacillus* strains have been recognized as probiotics (when the initial origin is human) or as beneficial microbes (when the origin is from foods) (Hill *et al*., 2014). They produce a wide range of functional foods, nutraceuticals, pharmaceuticals, and other health products with enhancing benefits for its physiological benefits throughout the *in-situ* production of diacetyl, oxygen peroxide and peptides with different antimicrobial, antioxidant, hypoglycemic and antihypertensive properties (Mada *et al*., 2019; Plavec and Berlec, 2019). *Lactobacilli* are extremely tolerant of diverse technological stress factors (Melgar-Lalanne *et al*., 2014), which have previously explored in lactic acid bacteria (LAB) strains (Silva *et al*., 2019; Papadimitrou *et al*., 2016).

Temperature is the main factor that rules lactic acid fermentation (Yuwono and Kokugan, 2008; Mostafaie *et al*., 2018) and may affect the production of secondary metabolites (Ahmed *et al*., 2006). Mesophilic temperatures (between 30 to 37 ºC) regulate the milk enzymatic coagulation for cheesemaking mean thermophilic temperatures (between 37 to 45 ºC) influence the milk acid coagulation for fermented milks (Tamime, 2002). Some lactic fermented foods may show bioactive and healthy properties, which are considered good vehicles for probiotics delivery (Demers-Mathieu *et al*., 2015). The effect of thermal stress on the survival of LAB has been studied at hot temperatures (>50 ºC) (Kulkarni *et al*., 2018; Ferrando *et al*., 2016). The growth of *L. reuteri* at 46 °C also increased survival during the freeze-drying of the strain compared with the normal growth at 37 ºC (Liu *et al*., 2014). However, despite its importance, the growth temperature has been rarely related to differences in the production of lactic acid and other metabolites of interest (Ferrando *et al*., 2015; Ferrando *et al*., 2016).

The predictive microbiology assesses the growth and behaviour of LAB, because of their adequacy to determine bacteria' kinetics such as growth and survival under diverse conditions (Cabral *et al*., 2019). So, mathematical modelling is a suitable tool to better select beneficial strains, design the equipment and determine the control parameters to ensure the final quality of the foodstuff (Tjørve and Tjørve, 2017). Nevertheless, the kinetic predictive modelling of some of the strains studied here is limited or even inexistent (Álvarez *et al*., 2010). The biomass can be used to derive the kinetic growth parameters (maximum specific growth rate, generation time and the adaptation phase period).

The characterization of the effects of time and temperature on antioxidant and hypoglycemic activity is essential to assess the optimal production of these metabolites (Li *et al*., 2012). Antioxidants are chemicals that can convert reactive species into stable products and decrease the adverse effects of ROS (Sah *et al*., 2016). Many *L. pentosus* strains isolates from diverse food environment (goat milk, cheese, kimchi) have shown antioxidant activity (Shu *et al*., 2017). However, few researches have been published with *L. pentosus* and *L. acidipiscis* (Shokryazdan *et al*., 2018). Finally, *Lactobacillus* spp. may regulate the glycaemic control via satiety signalling, gut integrity, and antioxidant protection of pancreatic cells (Tinderencel *et al*., 2020). *Lactobacilli* should be able to produce α-amylase and α-glucosidase inhibitors (Frediansyah *et al*., 2019). Both enzymes are key to digest carbohydrates. α-amylase hydrolyzes the α-linked polysaccharides into oligosaccharides, and the α-glucosidase catalyzes the last step in the digestion of carbohydrates to absorbable monosaccharides, including glucose. So, the inhibition of both enzymes can slow down the liberation of absorbable monosaccharides from the diet and prevent the sudden rise of blood glucose levels (Gajbhiye *et al*., 2018). The inhibition activity against these both enzymes has been reported in some LAB strains, specifically in *L. plantarum* strains (Ayyash *et al*., 2019), but, to the best of our knowledge, not in *L. pentosus* and *L. acidipiscis*. This enzymatic inhibition has been related to the antioxidant compounds present and, in the specific case of *Lactobacillus*, with the presence of bioactive peptides (Mudgil *et al*., 2018).

Lactobacillus plantarum, *Lactobacillus pentosus* and *Lactobacillus acidipiscis* used here have been previously isolated from a salty-ripened Mexican tropical cheese (double cream Chiapas cheese). Moreover, their probiotic potential has also determined, including *in vitro* survival thought the gastrointestinal tract, adhesion to mucin, aggregation properties and antimicrobial activity against some pathogen strains (Melgar-Lalanne *et al*., 2013). Their high tolerance to NaCl, acidity and alkalinity, presence of bile salts, antibiotic resistance, and other antimicrobials have also proved (Melgar-Lalanne *et al*., 2014). Other bacteria from Chiapas cheese have shown proteolytic activity with a high amount of free essential amino acids and γ-aminobutyric acid, which are related to some health properties as antioxidant, antimicrobial, antihypertensive, and even hypoglycemic (González-González *et al*., 2019).

This work aimed to model the growth of *Lactobacillus* strains (*L. plantarum*, *L. pentosus* and *L. acidipiscis*) previously postulated as probiotics at usual fermentation temperatures (32, 37 and 42 ºC) and *in vitro* evaluate their antioxidant and hypoglycemic activity.

2 Materials and methods

2.0.1 Strains and culture conditions

Lactobacillus plantarum, *Lactobacillus pentosus* and *Lactobacillus acidipiscis* were kindly donated by Dr. Humberto Hernández-Sánchez (Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Ciudad de México, México). The strains had been previously isolated from a salty-ripened Mexican tropical cheese (double cream Chiapas cheese) (Morales *et al*., 2011) and proposed as potential probiotics (Melgar-Lalanne *et al*., 2013). All the three strains were stored in Man Rogosa and Sharpe (MRS) broth (peptone 10.0 g/L, yeast extract 5.0 g/L, meat extract 5.0 g/L, glucose 20.0 g/L, dipotassium phosphate 2.0 g/L, dibasic ammonium citrate 2.0 g/L, sodium acetate 5.0 g/L, manganic sulphate 0.050 g/L, magnesium sulphate 0.010 g/l, tween 80 1 ml/L; pH 6.5) under congelation (at -20 °C) with glycerol as cryoprotectant 30% (v/v) until used. Previously to each experiment, strains were activated at least twice at 37 °C for 24 h in MRS broth.

2.1 Culture media and reagents

Salts, acids, alkalis, and chemical indicators used were ACS-grade. The rest of the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). MRS broth and MRS agar were purchased by Difco (Franklin Lakes, NJ, USA).

2.2 Experimental design

Experiments were carried on at three different fermentation temperatures: 32, 37 and 42 °C for 24 h in at least two independent experiments done by triplicate (n=6). These temperatures were decided according to the technological process in which *Lactobacillus* strains are usually involved, as previously explained. Growth parameters were expressed in absorbance at 590 nm (Spectrophotometer UV/vis; Cientifica Vela Quim, Mexico City, Mexico), biomass (g/L) and/or colony-forming units (CFU)/mL. Titratable acidity was expressed in lactic acid production (g/L). pH was also determined. All these parameters were experimental measured each 2h for 24 h in all the conditions tested with the three strains. A sterile and uncultivated MRS broth was used as a negative control in all the experiments.

The microbial growth in CFU/mL was determined with the correlation with the absorbance (590 nm) was done with an R>0.98 (data not shown). The CFU/mL was determined by the microdot technique (López de Lacey *et al*., 2014), and the biomass (g/L) was estimated by obtaining the dry weight biomass value estimated from 5 ml of freeze-dried samples (Álvarez *et al*., 2010).

2.3 Kinetic considerations for the prediction of the microbial growth

The unified version of the traditional 3-parameters Gompertz (U-Gompertz) model proposed by Tjørve and Tjørve, 2017 was used for the present modelling as equation (1):

$$W(t) = A\exp\left(-\exp(-e\times k_u(t-t_i))\right) \quad (1)$$

where $W(t)$ is the expected cell biomass as a function of time; A is the upper asymptote, k_u is the relative maximum growth coefficient; t_i is the time at the initial point and t is the time.

The absolute growth rate, $K(h^{-1})$, was given by Equation (2):

$$K(h) = A\times k_u \quad (2)$$

The resistance index (RI) was determined as Equation (3):

$$RI = \log\left(\frac{N_0}{N_t}\right) \quad (3)$$

where N_0 is the final growth (24h) expressed in CFU/mL at 37 ºC and N_t is the final growth expressed in CFU/mL at temperature at the end of the fermentation.

The Arrhenius Law describes the temperature dependence of the specific reaction rate constant in chemical reactions and it is still used as a secondary growth model in quantitative microbiology to determine the relationship between the temperature and the maximum specific rate (De Silvestri *et al*., 2018). The activation energy was obtained by linear regression as Equation (4):

$$\ln(K) = \ln(A) - \frac{E_a}{RT} \quad (4)$$

where A is a constant for each chemical reaction date defines the rate due to frequency of collisions (the upper asymptote); K it the absolute growth rate; E_a is the activation energy (J/mol); T is the absolute temperature (K), and R is the gas constant (8.314J/molK). The factor $\frac{E_a}{RT}$ is the probability that any given collision resultant in a reaction.

2.4 Antioxidant activity

The antioxidant activity of the strains at the initial time (0 h), at the end of the log or growth phase (14 h) and during well established stationary phase (24 h) was determined. The cell-free supernatants (CFS) were prepared by centrifugation (ThermoOECenctraCL2, USA) (8,000 x g, 15 min, 4 ºC) and then sterilized by using a 0.45 μm filter (Whatman, USA) and stored under -40 ºC until assays were performed. CFS was obtained at the initial time (0h), exponential growth phase (14 h) and stationary phase (24 h). The total phenolic compounds were determined by the Folin test (Guadarrama-Lezama *et al*., 2012). Results were expressed as gallic acid equivalents (μg) per mL of CFS.

The determination of radical scavenging activity by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was performed according to Ayyash *et al*. 2018. The DPPH reagent (0.1 mM) was dissolved in 95% methanol. Then, 200 μL of each sample (CFS) was shaken in vortex with 800 μL of the DPPH reagent and incubated in the dark at 25 °C for 30 min, and finally, the samples were read at 517 nm.

For the trolox equivalent antioxidant capacity (TEAC) assay, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was prepared according to Miliauskas *et al*., 2004 and read at 734 nm. Trolox was used as a standard. Data were expressed as μmol Trolox equivalent (TE)/mL.

2.5 Hypoglycemic activity

The α-amylase inhibition assay was performed as Ayyash *et al*. 2018, with few modifications. So, Type IV α-amylase from porcine pancreas (≥ 5 units/ mg) was prepared before the experiments with distilled water (0.5 units/mL). After that, 100 μL of each CFS was mixed with 100 μL of the α-amylase solution, and the mixture was incubated at 37 °C for 5 min. Then, 250 μL of corn starch (1%, w/v) was added as a substrate in phosphate buffer at pH 6.8 to start the reaction, and the mixture was incubated at 37 °C for 5 min. The reaction finished with the addition of 200 mL of DNS (1% 3,5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M NaOH), which was used as colouring reagent. The mixture was then heated at 100 °C for 15 min and diluted with 5 mL of distilled water.

The α-amylase activity was measured at 540 nm. The control was done with a solution without the CFS, and the blank was used with a starch solution. The inhibition percentage was determined as Equation (5).

$$Scavering\ rate\% = \left(1 - \frac{A_{sample} - A_{blank}}{A_{control}}\right) \times 100 \quad (5)$$

The α-glucosidase inhibition assay was performed as Mendoza-Avendaño *et al*. 2019, with few modifications. Briefly, 25 μL of α-glucosidase (1.0 units /mL) were dissolved in 50 μL 0.2 M potassium phosphate buffer (pH 6.8). Then, 50 μL of CFS were mixed with the previous enzymatic solution, and the mixture was incubated at 37 °C for 10 min. After that, 50 μL of 5mM pNPG (p-4-Nitrophenyl β-D-glucopyranoside) was added and incubated for 30 min at 37 °C. The reaction finished by adding 2 mL of 0.1 M Na_2CO_3. The enzymatic activity was determined by measuring the p-nitrophenol release from pNPG at 400 nm. A solution without the CFS sample was used as a control. A solution without the substrate was used as a blank. The inhibition (%) was calculated as the previous Equation (5).

2.6 Statistical analysis

The results are presented as means ± standard derivation. Fermentations were independently repeated at least two times, and the analysis was repeated by triplicate in all cases for the robustness of the research (n=6). Two-way ANOVA was used to determine the statistical differences for the U-Gompertz model (time was not considered as a variable). Three-way ANOVA was carried out to investigate the effect of time, temperature and bacterial strain on the growth, antioxidant and hypoglycemic activity and their interaction over the parameters analyzed ($p \leq 0.05$). Mean comparations was performed by the Tukey test ($p \leq 0.05$) at the same mean type. A Pearson correlation test was carried out to find any correlation between parameters for the same man type (pH and titratable acidity; DPPH and ABTS test and, α-amylase and α-inhibition). All statistical analysis was performed using the SigmaPlot

14.0 software (Systat Software, Inc., Chicago, IL, USA) version.

3 Results and discussion

3.1 Experimental kinetics of Lactobacillus strains

The cell growth (g/L), pH reduction and lactic acid production (g lactic acid/L) of three endogenous *Lactobacilli* isolated from Chiapas cheese (*L. plantarum*, *L. pentosus* and *L. acidipiscis*) in MRS broth were evaluated for 24 h at three different temperatures (32, 37 and 42 ºC) (figure 1).

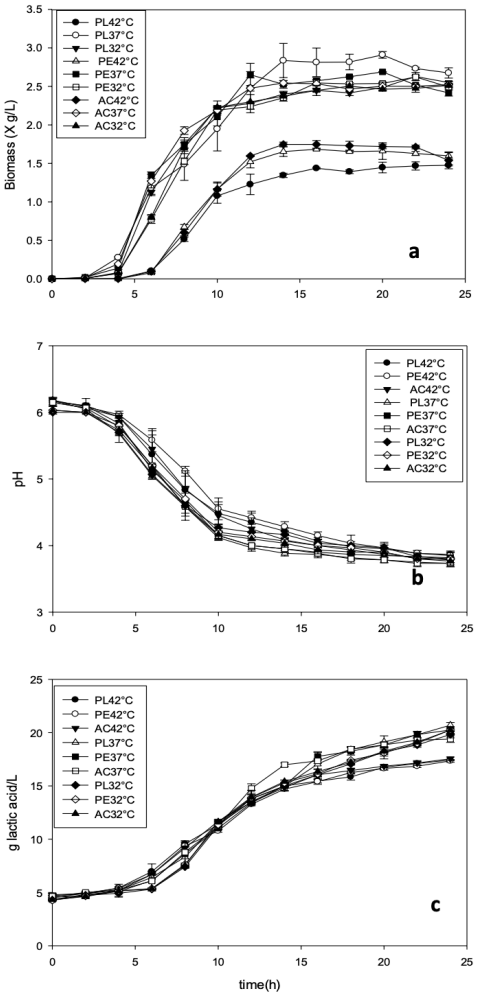

Fig. 1. Kinetic of *Lactobacillus plantarum, L. pentosus* and *L. acidipiscis* at 42, 37 and 32 °C for 24 h expressed in a) biomass production (g/L); b) pH; c) titratable acidity (g lactic acid /L) (n=6).

The cell growth was the factor that changed the most with the temperature (figure 1). The maximum growth was given at 37 ºC in the three strains tested, obtaining between 2.52 ± 0.0369 g/L (1.40×10^8 CFU/mL) in *L. acidipiscis* to 2.47 ± 0.0395 g/L (1.13×10^8 CFU/mL) in *L. pentosus* (figure 1a). The differences between 37 and 32 ºC were not significant ($p>0.05$), but all the strains showed significant differences with 42 ºC ($p<0.05$). Moreover, with all the strains significant lower results were obtained at 42 °C between 1.55 ± 0.04 g/L (1.83 × 107 CFU/mL) in *L. pentosus* to 1.54 ± 0.109 g/L (2.05 × 107cfu /ml) in *L. acidipiscis*. The three-way ANOVA showed significant differences between strains ($p=0.948$), but not between temperature ($p<0.01$) or time ($p<0.01$).

During the fermentation, bacteria consume the glucose of the medium and produce an increase in the concentration of hydrogen ions (H_3O^+) and titratable acidity (figures 1b and 1c). These biochemical changes were negligible during the first three h of the fermentation (adaptation phase), with a significant increase during the exponential growth phase of bacterial growth. The titratable acidity (g lactic acid/L) increased with the fermentation time and was lower at the highest temperature tested. When three-way ANOVA was done, no significant differences were found between the three strains ($p=0.077$) but, significant differences with the temperature ($p < 0.01$) and time ($p < 0.01$) were found. The maximum of lactic acid was produced by *L. pentosus* at 37 °C (20.70 ± 0.27 g/L). The lactic acid production depended on the strain, the temperature applied, and the primary source of carbohydrates in the medium (Zotta *et al.*, 2017). The pH resulted in temperature and time-dependent ($p<0.01$), but the influence of strain was not clear ($p=0.003$). As expected, an inverse Pearson correlation between pH and titratable acidity ($P=-0.938$) was found; equally, it was reported in other *Lactobacillus* strains (O'Hanlon *et al.*, 2013). Neither a linear Pearson correlation between titratable acidity nor bacterial growth was found ($P=0.877$).

3.2 Modelling the kinetic growth

In this study, the growth kinetics of *L. plantarum*, *L. pentosus* and *L. acidipiscis* cultured at different temperatures (32, 37 and 42 °C) in the function of time was investigated using the U-Gompertz model recently proposed by Tjørve and Tjørve, 2017 as a modification from 3-parameter Gompertz equation. The Gompertz model has been widely used in predictive microbiology (Peleg and Corradini, 2011).

Table 1. Absolute growth rate, resistance index at 24 h of *L. plantarum, L. pentosus* and *L. acidipiscis* and regression coefficient, using the U- Gompertz model.

Strain	T (°C)	K (h^{-1})	R_1	RI	Ea (J/mol*K)	R_2
L. plantarum	42	0.229 ± 0.001	0.996[a]	0.790 ± 0.056[a]	-55,375.21 ± 0.924	0.924
	37	0.414 ± 0.005	0.990[b]	*na*		
	32	0.460 ± 0.002	0.992[a]	0.012 ± 0.042[b]		
L. pentosus	42	0.294 ± 0.001	0.997[a]	0.789 ± 0.056[a]	-30,331.19 ± 0.680	0.680
	37	0.507 ± 0.003	0.987[b]	*na*		
	32	0.294 ± 0.001	0.995[a]	0.012 ± 0.042[b]		
L. acidipiscis	42	0.400 ±0.001	0.990[a]	0.829 ± 0.058[a]	-18,298.49 ± 0.773	0.773
	37	0.526 ±0.001	0.996[b]	*na*		
	32	0.504 ± 0.007	0.996[b]	0.013 ± 0.044[b]		

Different superscript letter for the same strain indicates significant difference ($p<0.05$). K. Absolute growth rate, R_1: Correlation coefficient in the U-Gompertz model; RI: resistance index; R_2: Regression coefficient for Activation Energy (E_a), *na*: not apply. Experiments were done at 32, 37 and 42 °C and 0, 14 and 24 h in MRS broth. n=6.

Moreover, some modifications of the Gompertz model have been successfully applied to measure the growth of different microbial growth (García-Sánchez *et al*., 2019) and specifically the growth of *Lactobacillus plantarum* strains (Juárez-Tomás *et al*., 2002; Demir *et al*., 2006) and *L. pentosus* (Rodríguez-Gómez *et al*., 2012) but, to our knowledge, not in *L. acidipiscis*. The model could successfully describe the growth curve of *Lactobacilli* strains (Juárez-Tomás *et al*., 2002). The U-Gompertz model is a recent modification of the traditional 3-parameters Gompertz model that allows us to unify and simplify the interpretation of growth rates (Tjørve and Tjørve, 2017), but to our knowledge, it has not been used to model the growth of LAB.

Data from the absolute growth rate, the regression coefficient of the U-Gompertz model and the RI are shown in table 1. The experimental growth kinetics were obtained in the laboratory with the measurement of the absorbance (590 nm), the CFU/mL and biomass production (g/L) by correlation. The biomass production was decided to be used to determine the absolute growth rate (h^{-1}) as well as the regression coefficient (R_1) in the kinetic model by the application of the modified U-Gompertz model.

The two-way ANOVA analysis showed that the absolute growth rate at the end of the 24 h of fermentation (K) was independent of the strain ($p=0.115$), but not from the temperature applied ($p=0.035$), and ranked from 0.229 h^{-1} in *L. plantarum* at 42 ºC to 0.526 h^{-1} in *L. acidipiscis* at 37 ºC, and no interactions were found between the parameters. Moreover, the greatest changes in the absolute growth rate (K) were given in the *L. plantarum* strain ranking from 0.229 ± 0.01 h^{-1} (at 42 °C) to 0.460 ± 0.02 h^{-1} (at 32 °C) being the strain more negatively affected by the highest temperature. This result confirms the mesophilic character of *L. plantarum* strain. The RI was determined considering 37 ºC as the optimum temperature for bacterial growth. So, bacteria tested here were able to grow at 32, and 37 ºC without significant differences in their growth ($p>0.05$) and significant differences in the temperature of growth was given at 42 ºC ($p>0.05$) which could be considered as a stressful condition for the bacteria. Then, the RI ranked from 0.012 ± 0.042 in *L. plantarum* and *L. pentosus* at 32 °C to 0.829 ± 0.058 in *L. acidipiscis* at 42 °C. Result obtained here were like previous works which consider 37 °C as the ideal temperature for some *Lactobacillus* spp, especially *L. pentosus* strains (Asteri *et al*., 2009; Ferrando *et al*., 2015).

The results of RI, together with the growth kinetics of the strains at the temperatures analyzed, demonstrated the mesophilic character of the three *Lactobacillus* isolated from Chiapas cheese. Similar behaviour has been previously reported in *L. acidophilus*, *L. rhamnosus* GG, and *L. reuteri* strains,

which dramatically decreased at 45 ºC (Østlie *et al*., 2005). The mesophilic character of some *Lactobacillus* strains had been previously tested on the bases of their growth and isolation at mesophilic temperatures (between 25-37 °C) in the case of *L. plantarum* (Demers-Mathieu *et al*., 2015) and *L. pentosus* strains (Mannu *et al*., 2000), and *L. acidipiscis* (Asteri *et al*., 2009). However, the temperature interval of growth has been poorly analyzed in *L. pentosus* and *L. acidipiscis*. Moreover, the growth phase could be experimentally determined. So, the lag or adaptation phase occurred during the first four hours at 42 °C in all the strains but only lasted two hours at 37 and 32 °C. The exponential growth phase ended at 14 h at 42 °C, at 16 h at 37 °C and, at 18 h at 32 °C. Finally, the stationary phase started between 14 and 16 h and could be well consolidated in all the cases at 24 h. This analysis was important to determine the times for the antioxidant and hypoglycemic activity at the different growth phases, which may have a technological importance at the industrial level. The robustness of the U-Gompertz kinetic model chosen was defined by the regression coefficient which was minimum in *L. pentosus* at 37 °C (R= 0.987) and could be considered as adequate to represent the biomass increase as a function of the fermentation time and temperature (Zwitering *et al*., 1990). However, all the strains could grow almost equally at 32 than at 37 °C, but the growth was significantly reduced at 42 °C showing the mesophilic character of three strains tested. So, the temperature was a significant factor (p=0.006) but not the strain. Results obtained indicated that the growth at 32 °C positively contributes to the proliferation of the microorganisms during the fermentation reducing the economic cost of the process, which could be an important economic issue at the technological level (Barãoa *et al*., 2019).

Linear Arrhenius models have used to estimate the sensitivity at a temperature in freeze-dried LAB (Yao *et al*., 2008), the lower temperature in which microbial life is possible (-17 ºC) (Price and Sowers, 2004), the shelf life in corn yogurt (Aini *et al*., 2017), and the effect of temperature over the growth rate in some LAB strains (Adamberg *et al*., 2003). The absolute growth rate calculated allowed us to calculate the empirical Arrhenius equations, as shown in table 1. The E_a factor varied from -18,298.49 ± 0.773 J/mol*K in *L. acidipiscis* to -55,375.21 ± 0.924 J/mol*K in *L. plantarum*. However, the lineal Arrhenius model is weak to explain the effect of temperature on the growth behaviour of the strains as previously demonstrated (Ibarra *et al*., 2012) because the inclusion of the Universal Gas Constant may not explain the microbial growth kinetics properly (Peleg *et al*., 2011). So, a more precise model should be developed to explain the thermodynamic variables implied in the bacterial growth.

3.3 Antioxidant activity

DPPH and ABTS free radical scavenging ability have been widely used to determine the antioxidant activity of foods (Gülçin 2011). In the present research, the antioxidant activity of the endogenous *Lactobacilli* isolated from Chiapas cheese is shown in table 2. As expected, all strains tested did not produce polyphenols and so, data are not shown. When the DPPH test was done, the three strains only showed differences in the time ($p<0.01$), but not in the strain ($p=0.607$) or the temperature applied ($p=0.008$). However, in the ABTS test, all the factors showed statistical differences ($p<0.01$). Moreover, statistical differences in the interaction between the three factors analyzed were found ($p<0.001$), which indicates that the effect of one factor is not consistent with all the combinations of the two other factors. Moreover, as expected, no Pearson correlation between both methods was found ($P=0.450$). Other authors (Venegas-Ortega *et al*., 2020; Shi *et al*., 2019) have analyzed the antioxidant activity of presumptive LAB isolated from cheeses. However, these studies can not be comparable to the presented here because the unit used to express the results, the incubation time and/or the broth media used are different.

It is essential to mention that bioactive compounds in fermented foods significantly contribute to the alleviation of reactive oxygen species such as superoxide, hydroxyl, and peroxyl radical formed by oxidatively stressed cells. These reactive species are related to a wide variety of physiological and pathogenic processes (cell proliferation, cell differentiation, apoptosis, and neurodegenerative disorders between others), and their prevention is considered as a health priority (Ayyash *et al*., 2018). The increase of the antioxidant activity with the time is probably related to the proteolytic activity of the strains as in other *Lactobacillus* strains occurs (Vaštag *et al*., 2010).

Table 2. Antioxidant activity quantified by the DPPH and ABTS+ assays (μM Trolox/mL) and hypoglycemic activity quantified as α-amylase inhibition (%) and α-glucosidase inhibition (%).

Strain	T (°C)	Time (h)	Antioxidant activity DPPH (μM Trolox/mL)	Antioxidant activity $ABTS^{+}$ (μM Trolox/mL)	Hipoglucemic activity α-amylase (Inhibition %)	Hipoglucemic activity α-glucosidase (Inhibition %)
L. plantarum	32	0	4509.66 ± 318.52	1051.85 ± 163.89	4.258 ± 0.926	1.973 ± 0.360
		14	5184.69 ± 497.25^{C}	1478.93 ± 77.83^{A}	73.650 ± 1.539 ^{A}	61.829 ± 1.674 ^{A}
		24	6980.73 ± 438.44^{A}	1529.05 ± 61.70^{A}	90.286 ± 1.809 ^{A}	99.088 ± 0.506 ^{A}
	37	0	3708.07 ± 574.05	1209.83 ± 44.38	3.429 ± 0.304	2.176 ± 1.496
		14	2219.40 ± 311.25^{B}	1296.99 ± 130.10^{C}	86.637 ± 1.872 ^{A}	93.140 ± 0.559 ^{A}
		24	5058.12 ± 759.47^{A}	1226.17 ± 55.69^{C}	95.686 ± 0.745 ^{A}	99.489 ± 0.212 ^{A}
	42	0	1930.11 ± 522.89	1379.79 ± 93.39	4.302 ± 0.681	1.973 ± 0.360
		14	5480.01 ± 389.06^{A}	1389.60 ± 140.75^{C}	52.310 ± 2.810 ^{A}	61.829 ± 1.674 ^{A}
		24	4877.31 ± 623.55^{A}	1364.54 ± 39.76^{C}	77.714 ± 2.066 ^{A}	99.088 ± 0.506 ^{A}
L. pentosus	32	0	4642.26 ± 349.83	1281.73 ± 63.24	4.059 ± 1.443	2.273 ± 1.156
		14	7402.62 ± 318.52^{A}	1361.27 ± 69.57^{C}	75.173 ± 1.221 ^{A}	85.304 ± 0.840 ^{A}
		24	5305.23 ± 533.42^{C}	1460.41 ± 36.15^{A}	95.600 ± 1.207 ^{A}	99.560 ± 0.419 ^{A}
	37	0	1580.54 ± 544.03	1098.70 ± 44.50	3.933 ± 2.177	3.172 ± 1.480
		14	3870.80± 348.73^{A}	1300.26 ± 59.58^{A}	70.272 ± 1.883 ^{A}	98.668 ± 0.454 ^{A}
		24	5642.74 ± 25.57^{A}	1336.21 ± 79.82^{A}	94.984 ± 0.709 ^{A}	99.735 ± 0.131 ^{A}
	42	0	3328.37 ± 595.30	1407.03 ± 57.27	3.487 ± 1.292	2.092 ± 1.297
		14	6516.65 ± 570.15^{A}	1488.74 ± 141.42^{C}	51.950 ± 1.043 ^{A}	85.379 ± 1.024 ^{A}
		24	6064.63 ± 479.86^{A}	1195.66 ± 45.80^{B}	79.517 ± 2.495 ^{A}	95.955 ± 0.574 ^{A}
L. acidipiscis	32	0	3280.15 ± 570.72	1181.50 ± 97.45	4.131 ± 0.503	2.414 ± 1.528
		14	5269.06 ± 418.74^{A}	1295.90 ± 101.95^{C}	79.336 ± 3.092 ^{A}	94.382 ± 0.450 ^{A}
		24	6793.89 ± 552.09^{A}	1612.94 ± 56.71^{A}	96.455 ± 1.083 ^{A}	96.616 ± 0.282 ^{A}
	37	0	3388.64 ± 290.61	981.03 ± 47.29	4.240 ± 0.790	2.575 ± 1.489
		14	4256.53 ± 451.78^{A}	1124.85 ± 92.47^{C}	66.727 ± 2.046 ^{A}	98.469 ± 0.651 ^{A}
		24	4569.93 ± 244.15^{A}	1153.17 ± 79.01^{A}	97.084 ± 0.752 ^{A}	99.756 ± 0.104 ^{A}
	42	0	4901.42 ± 933.76	1018.07 ± 78.65	3.377 ± 1.538	1.774 ± 0.340
		14	4087.77 ± 525.28^{C}	1403.76 ± 82.49^{A}	41.963 ± 1.067 ^{A}	84.764 ± 0.925 ^{A}
		24	7125.38 ± 751.83^{A}	1341.66 ± 112.80^{A}	81.759 ± 1.964 ^{A}	99.644 ± 0.273 ^{A}

*Significant difference ($p< 0.05$) for the antioxidant and hypoglycemic activity with respect to the initial time (0 h); A, when the antioxidant or hypoglycemic activity has significantly increased; B, when the antioxidant or hypoglycemic activity has significant decreased; C, when there is no significant difference. Experiments were done at 32, 37 and 42 °C and 0, 14 and 24 h in MRS broth. *n=6.*

3.4 Hypoglycemic activity

The inhibition of α-amylase and α-glucosidase is considered as a practical biochemical approach to hypoglycemic and are used as hypoglycemic agents to treat the diabetes disorder. The inhibition of these both enzymes could result in postponing the digestion, absorption of starch and can reduce the postprandial hyperglycemia. There are some synthetic

inhibitors, but they can cause some intestinal disorders (Ragul *et al*., 2020). Some *Lactobacillus* strains can produce amylase to hydrolyze small quantities of starch from vegetable sources, and glucosidases to hydrolase starch and disaccharides to glucose during the fermentation (Behera *et al*., 2012). It is essential to mention that the lactic fermentation of vegetables is traditionally given at a mesophilic range of temperatures. However, at physiological temperatures, some *Lactobacillus* strains can inhibit α-amylase action. This inhibition has been related to the presence of bioactive peptides, produced for the hydrolysis of the peptones presents in the MRS broth and the hydrolysis of milk proteins during the fermentation (Ayyash *et al*., 2018).

The inhibition results of α-amylase and α-glucosidase of *L. pentosus*, *L. pentosus* and *L. acidipiscis* at different temperatures and growth phases are illustrated in table 2. As expected, before the fermentation, the medium has no shown α-amylase and α-glucosidase inhibition activity. So, the inhibition was due to the *Lactobacillus* strains fermentation process. The inhibition increased during the fermentation time, which indicates the possibility of a secretion of bioactive metabolites by the bacteria. The inhibition of α-amylase resulted in strain ($p=0.05$), temperature ($p<0.01$) and time ($p<0.01$) dependence mean the inhibition of α-glucosidase resulted in statistical differences with the three factors ($p<0.001$). In general, the highest inhibition of both enzymes was obtained at 37 °C and 24h, which is considered the human physiological temperature. A good Person correlation was found between both enzymes ($P=0.963$) like previous work with bovine milk and *Lactobacillus plantarum* strains (Ayyash *et al*., 2018). In *L. pentosus* strains, the inhibition results of α-amylase and α-glucosidase have also been reported (Frediansyah *et al*., 2019). However, previous reports have been done in milk and vegetable sources, not in MRS broth, which is considered the election media from *Lactobacillus* strains. The results showed here demonstrated that *L. acidipiscis* had a high inhibition activity against α-amylase and α-glucosidase even greater than *L. pentosus* and *L. pentosus*. Moreover, the best results were obtained at the physiological temperature (37 °C), which may imply and antidiabetic physiological effect in the gastrointestinal tract. So, *in vivo* studies about the potential antidiabetic effect of this strain may be done to corroborate this finding. The antidiabetic activity in the cheese has been related to the production of bioactive peptides, although the peptides were not sequenced (Mushtag *et al*., 2019). So, the effect of the addition of probiotic *L. plantarum, L. casei* and *L. brevis* to Kalari cheese have been tested, finding a higher inhibition % in the cheese added with probiotic *Lactobacillus* (Mushtag *et al*., 2019).

Conclusions

The results presented here suggest the relevance of the technological temperature and growth phase when select potential probiotic strains on the viability and functional properties of the strains. Moreover, to our knowledge, this is the first report about the influence of temperature and growth phase over the *in vitro* antioxidant and hypoglycemic activity in potential probiotic *L. plantarum*, *L. pentosus* and *L. acidipiscis* isolated from a salty-ripened Mexican tropical cheese (double cream Chiapas cheese). The growth, antioxidant activity and hypoglycemic activity resulted in time and temperature dependence and the growth could be successfully modelled ($R>0.987$) with the U-Gompertz equation. The temperature range for the growth of the strains was 32-37 °C. So, the strains could be considered mesophilic. To the best of our knowledge, this is the first report which mentions the mesophilic character of an *L. acidipiscis* strain. Furthermore, this research also determined, for the first time, the *in vitro* antioxidant and hypoglycemic activity of *L. acidipiscis*. The functionality of the strains at different temperatures by *in vivo* models is currently being analyzed by our research group.

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