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favecv@gmail.com

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RESEARCH ARTICLE

Low-cost culture medium for biomass production of lactic acid bacteria with probiotic potential destined to broilers

Berisvil AP¹, Astesana DM¹, Zimmermann JA¹, Frizzo LS^{1,2}, Rossler E¹, Romero-Scharpen A¹, Olivero CR¹, Zbrun MV^{1,2*}, Signorini ML^{2,4}, Sequeira GJ², Drago SR⁵, Soto LP^{1,2}

¹ Laboratorio de Análisis de Alimentos, Instituto de Ciencias Veterinarias del Litoral (ICIVET Litoral), Universidad Nacional del Litoral - Consejo Nacional de Investigaciones Científicas y Técnicas (UNL-CONICET), Esperanza, Santa Fe, Argentina.

² Departamento de Salud Pública, Facultad de Ciencias Veterinarias (FCV), UNL, Argentina.

³ CONICET, Argentina.

⁴ Instituto Nacional de Investigación Agropecuaria - Estación Experimental Agropecuaria Rafaela, Argentina.

⁵ Instituto de Tecnología de los Alimentos, Facultad de Ciencias Químicas, UNL, Argentina.

* Correspondence: María Virginia Zbrun, ICIVET Litoral (UNL - CONICET), RP Kreder 2805 (CP 3080) Esperanza, Santa Fe, Argentina. E-mail: virginiazbrun@yahoo.com.ar

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SUMMARY. The aim of this study was to evaluate different low-cost culture media for biomass production of 3 potential probiotic *L. salivarius* strains, which could be destined to broilers at farms. Different formulated media based on whey permeate (WP) supplemented with nitrogenous sources were evaluated: yeast extract (YE), whey hydrolysate (WH) and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (Mn), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Mg). The growth of each strain in the formulated media and the cost was compared with their growth and cost in commercial medium (MRS). *L. salivarius* DSPV008P did not grow adequately in any of the formulated media. On the other hand, addition of YE and Mn in the formulated media increased *L. salivarius* DSPV002P and *L. salivarius* DSPV011P growth. In contrast, WH and Mg addition increased the *L. salivarius* DSPV002P biomass only. *L. salivarius* DSPV011P was the only strain that had similar growth performance in MRS as in the selected medium: WP + YE 8g/l + Mn. In this sense, *L. salivarius* DSPV011P reached a biomass of 9.22 Log (CFU/ml) in the selected formulated medium, with a low-cost growth medium 12 times less than in MRS. Although the effect of supplements added to the culture medium on kinetic parameters are strain dependent, *L. salivarius* DSPV011P is the strain with the best technological characteristics, capable of growing in a medium based on a by-product of the dairy industry supplemented with YE and Mn and at a much less cost than in MRS medium.

RESUMEN. Desarrollo de un medio de cultivo de bajo costo para la producción de biomasa potencialmente probiótica destinado a pollos parrilleros. El objetivo de este trabajo fue evaluar diferentes medios de cultivo de bajo costo para la producción de biomasa de 3 cepas potencialmente probióticas de *L. salivarius*, las cuales podrían ser destinadas a pollos parrilleros en las granjas. Para ello se evaluaron diferentes formulaciones basadas en permeado de suero de queso (WP) suplementado con fuentes nitrogenadas: extracto de levadura (YE) e hidrolizado de suero (WH) y $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (Mn), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Mg). El crecimiento de las cepas en estas formulaciones y el costo económico fue comparado con el crecimiento y costo en el medio de cultivo comercial (MRS). *L. salivarius* DSPV008P no creció adecuadamente en ninguno de los medios evaluados. Por otro lado, la adición del YE y Mg al medio mejoró el desarrollo microbiano de *L. salivarius* DSPV002P y *L. salivarius* DSPV011P. El agregado de WH y Mn solo tuvo un efecto positivo en el incremento de la biomasa de *L. salivarius* DSPV002P. *L. salivarius* DSPV011P fue la única cepa que desarrolló la misma cantidad de biomasa en MRS y en el medio seleccionado WP + YE 8 g/L + Mn. *L. salivarius* DSPV011P logró un desarrollo de biomasa de 9.22 Log (UFC/ml) en el medio seleccionado con un costo económico 12 veces menor que en MRS. Aunque el efecto de los suplementos añadidos al medio de cultivo sobre los parámetros cinéticos depende de la cepa, *L. salivarius* DSPV011P es la cepa con mejores características tecnológicas, capaz de crecer en un medio a base de un subproducto de la industria láctea suplementado con YE y Mn y a un costo mucho menor que en MRS.

Keywords: probiotic, *Lactobacillus salivarius*, whey permeate, biomass production

Introduction

In broiler farms, the environment, management measures and nutrition can modify the intestinal microbial conditions and facilitate infection by pathogens (Scallan *et al.*, 2011). Pathogenic micro-organisms could cause morbidity and mortality resulting in enormous economic losses in the poultry production (Hossain *et al.*, 2017).

The use of antibiotics in feed intended for poultry to prevent disease is usually a common practice. In this sense, antibiotics are used at sub-therapeutic doses as growth promoters in order to improve production performance (Bedford *et al.*, 2000; Hung *et al.*, 2012). However, the current trend is to avoid the use of antibiotics as growth promoters due to the concern for the emergence of resistant strains. In Argentina, the National Service of Agrifood Health and Quality banned the use of antibiotics as growth promoters since 2019 (SENASA, 2015). Therefore, it is necessary to evaluate alternative tools which provide a solution to this problem. Probiotics emerge as an alternative to anti-biotic, they are used to prevent diseases or as growth promoters. Probiotics were defined as live micro-organisms that, administered in adequate amounts, confer a beneficial effect on the health of the host (FAO, 2001). Balanced microbiota of gastrointestinal tract can provide efficient protection against pathogenic microorganisms, leading to better animal performance (Hung *et al.*, 2012; Niba *et al.*, 2009).

The viability and quantity of microorganisms at the time of inoculation are of vital importance to produce the probiotic effects. The probiotic exerts their effect if they are viable and at a minimum concentration of 10^6 CFU/ml (Vinderola *et al.*, 2000). Therefore, to produce a probiotic inoculum to supplement feed for broilers, the culture medium has to be optimized to obtain a high viable microorganisms yield with low cost raw materials.

Lactic acid bacteria (LAB) have been proposed as and are used as probiotic strains (Blajman *et al.*, 2017). Lactic acid bacteria (LAB) are a nutritionally demanding group of microorganisms. They need a wide range of nutrients to grow and synthesize metabolic products, and some nutritional requirements are usually strain specific (Hammes *et al.*, 2009). LAB are characterized by using different sources of carbon and produce mainly lactic acid as the final product of metabolism. According to the metabolism, LAB are classified as homo- and hetero-fermentative. *L. salivarius* is obligately homo-fermentative and has the ability to ferment lactose unlike other LAB species (Wood *et al.*, 1995).

There are commercial growth media formulated specifically for LAB such as MRS (De Man *et al.*, 1960). It contains macro and micronutrients specific to this

bacterial group. However, the large-scale production of *Lactobacillus* spp. in MRS is not profitable, so is interesting evaluate the use of by-products of the industry as low-cost growth medium. The by-products of the dairy industry could be used as alternative culture media to the MRS. In this sense, WP is a low-cost product used as growth medium of probiotic bacteria and it is an alternative input to obtain probiotic biomass in a more economical way (Hugo *et al.*, 2016).

WP is generally composed of lactose (70 - 90%), protein (0.5 - 3%) and could have traces of mineral salts such as phosphate, potassium, magnesium, sodium, calcium (Hu *et al.*, 2014; Trigueros *et al.*, 2016). However, with the aim to obtain an optimal microbial growth in WP, is necessary to add different nutrients according to the species or strains of LAB evaluated. In this sense, supplementation with nitrogenous sources and mineral salts is necessary and usually it is a limiting factor for the industrial production of biomass. The cost of supplementing the medium with YE can represent up to 38% of the final cost of biomass production (Altaf *et al.*, 2007). Its high cost affects the economic efficiency of the production of biomass, so it is important to reduce YE concentration or find economical alternatives of nitrogenous sources. For this purpose, WH could be used as a low cost additive to increase biomass production (Kwon *et al.*, 2000).

The supplementation of the medium with hydrolyzed proteins has the advantage of providing the bacteria with a more accessible nitrogen source and also, in some cases, with greater bioactivity (Dabrowska *et al.*, 2017). Likewise, positive effect of the addition of salts such as Mn and Mg on microbial mass growth of different BAL species has been demonstrated previously (Raccach *et al.*, 1985).

To evaluate the performance of LAB, the study of their kinetic parameters of growth are the basic tools to scale the biotechnological processes evaluated in the laboratory. This allows predicting the development of fermentation and evaluating the yields and productivities in the processes (De Man *et al.*, 1960). The most important parameters of microbial growth are: the maximum specific growth rate (V_{max}), the time it takes the bacteria to reach the maximum growth rate (TV_{max}), the lag time or Lag phase (Lag) and the maximum number of bacteria reached (N_{max}). The knowledge of the behavior of growth kinetic parameters, would allow to diminish the times and to increase the yields of the industrial processes.

Therefore, the aim of this study was to evaluate the growth of potential probiotic *L. salivarius* in low-cost media based on WP, supplemented with nitrogenous sources and mineral salts. This bacteria biomass could be used as feed supplement in broilers farms for

diseases prevention and improve the animal growth parameters.

Materials and methods

Microorganisms

The strains from avian origin were: *L. salivarius* DSPV002P, *L. salivarius* DSPV008P and *L. salivarius* DSPV011P. They belong to LAA ICiVet / UNL-CONICET and were selected for their resistance to gastro-intestinal conditions, cell surface hydrophobicity and production of antimicrobial compounds (Blajman *et al.*, 2015). These strains were qualified for further evaluation as potential probiotics. Their GenBank access numbers are KU295171, KU295177 and KU295180, respectively. The strains were maintained in a cryo-protective medium based on tryptone broth with glycerin (17% v/v) and sterile equine serum (5% v/v) at -80 °C until use.

Preparation of WH

WH was obtained using 2 L batch thermostatic reactor. The enzymatic hydrolysis parameters were selected according to manufacturer's guidelines and previous assays (Soto *et al.*, 2006). A dispersion of 6 g/100 g whey protein was prepared by dissolution in distilled water. Temperature (T) was brought to 55 °C in a water bath, during 20 min under gentle agitation. The reaction pH was adjusted to 6.0. Enzymatic digestion of substrate was performed at 55 °C, using Flavourzyme at enzyme/substrate ratio (E/S) of 5 g/100 g. The pH was continuously measured using an IQ Scientific Instruments pH-meter and adjusted by addition of 3 mol/L NaOH. Hydrolysis was performed for 4 h. Enzyme activity was stopped by heating the dispersion at 85 °C for 5 min. After inactivation treatment, the solutions were cooled to room temperature with tap water, and refrigerated at 4 °C until use in the experiments.

Free amino groups were measured using o-phthalaldehyde, according to Nielsen *et al.* (2001), and the degree of hydrolysis (DH) was calculated as:

$$DH = [(h - h_0) / h_{tot}] \times 100$$

Where: h_{tot} : is the total number of peptide bonds in the protein substrate (8.8 mEq/g protein); h : is the number of peptide bonds cleaved during hydrolysis, and h_0 : is the content of free amino groups in the substrate.

In this hydrolysis conditions, a WH with 6.3 % DH was obtained.

Formulated culture media

The WP was obtained from Arla Foods Ingredients S.A. (Córdoba, Argentina) and contained lactose (85% w/w), ashes (Na and Ca 6% w/w), and proteins (3% w/w) as declared by the manufacturer. It was used as a base

medium 60 g/L to formulate culture media, which were supplemented with YE (Oxoid, Basingstoke, United Kingdom), WH, Mn (Anedra, San Fernando, Argentina), Mg (Merck, Germany). The concentrations evaluated of each supplement were: YE (0 g/L, 4 g/L and 8 g/L), WH (0 g/L, 7 g/L and 14 g/L), Mn (0 g/L and 0.003 g/L), Mg (0 g/L and 0.02 g/L). A total of 36 culture media formulations were obtained as a result of all possible combinations of the different supplements and their concentrations.

Analysis of growth kinetic parameters

Strains were grown in MRS broth (Biokar, France) in two consecutive cultures for 24 h at 37 °C. Each strain was inoculated at 1% v/v (2.4 µL) in sterile 96-well microplates, which previously contained 240 µL of the aforementioned media formulations by triplicate. Optical density (OD = 630 nm) were measured on the Multi-Mode Microplate Reader, Synergy™ HT Biotek® for 24 h every 30 min. Incubation was performed for 24 h at 37 °C. MRS broth was used as control of optimal growth and each formulated medium without inoculum was used as a negative control. The results were analyzed using the Gen 5 software (Biotek®) and the following growth kinetic parameters were analyzed: Vmax, TVmax, Lag phase and Nmax. Selection of the medium formulated for each strain was based on the best results of the kinetics parameters.

Quantification of the growth of the strains in the selected media

Strains were grown in MRS broth (Biokar, France) in two consecutive cultures for 24 h at 37 °C to obtain a stock culture. Five ml of these stock cultures were inoculated in: a) 50 ml of the medium that showed the best growth kinetic parameter results of each evaluated strain, b) 50 ml of WP c) 50 ml of MRS broth. Media were incubated for 24 h at 37 °C in aerobiosis. After the incubation, serial dilutions were made in Ringer ¼ solution and counts in MRS agar were carried out to determine the colony forming unit count per milliliter (CFU/mL).

Economic cost of the formulated media

Economic cost of the selected culture medium, which produced the best kinetic parameters, was evaluated for each strain. The cost to produce 1000 L of each culture medium was calculated and compared to the cost of the same volume of MRS (Vázquez *et al.*, 2009). The costs of the selected media were calculated by adding the cost of each ingredient and the MRS cost was calculated from a commercial medium.

Growth of *L. salivarius* DSPV 011P on the selected formulated medium in a lab reactor scale

Lactobacillus salivarius was cultured in a 5 L (Sartorius Stedim Biotech, Goettingen, Germany) with 1% of initial inoculum obtained by two successive incubations during 24 h at 37 °C in MRS. Fermentation was done through a batch process during 18 h at 37 °C with 60 g/L WP, 8 g/L YE and 0.003 g/L MnSO₄ as culture medium.

Agitation was set at 120 rpm and pH was adjusted to 6. After the incubation, serial dilutions were made in Ringer ¼ solution and counts in MRS agar were carried out to determine the colony forming unit count per milliliter (CFU/mL).

Statistical analysis

To select the best medium for each strain studied, the best values of N_{max} and V_{max} were considered. The effect of supplements on N_{max} y V_{max} was analyzed using a complete factorial experimental design: 3 (YE: 0 g/L, 4 g/L and 8 g/L) x3 (WH: 0 g/L, 7 g/L and 14 g/L) x 2 (Mn: 0 g/L and 0.003 g/L) x 2 x Mg (0 g/L and 0.02 g/L) and Duncan test ($P < 0.05$). When two or more media showed similar values of N_{max} and V_{max} , a second analysis was performed among these media based on the parameters T_{vmax} and Lag phase by one-way ANOVA and Duncan's test.

Growth parameters of the selected media were compared with growth parameters of MRS and the basal medium (WP) for each strain by one-way ANOVA and Duncan's test. All statistical analysis were performed with the Statgraphics Plus for Windows program. 11.0. (Statgraphics Plus Software®, 1997).

All experiments were carried out in triplicates, with the mean values being reported.

Results

Selection of formulated media based on kinetics parameters

L. salivarius DSPV002P

The highest amount of biomass (N_{max}) was obtained when YE and WH were at 4 g/L and 8 g/L ($P < 0.001$), and at 7 g/L and 14 g/L ($P < 0.001$), respectively, and Mn at 0.003 g/L ($P < 0.001$) in the culture medium. In turn, Mg did not modify ($P = 0.284$) *L. salivarius* DSPV002P growth (Table 1).

On the other hand, the growth rate (V_{max}) of *L. salivarius* DSPV002P was the highest when YE and WH were at 4 g/L and 8 g/L ($P < 0.001$), and 7 g/L and 14 g/L ($P = 0.011$), respectively, and Mg ($P = 0.037$) were present in the medium. The presence of Mn did not influence the growth rate ($P = 0.814$) (Table 1).

Therefore, in order to obtain the greatest amount of biomass at the shortest time, both nitrogen sources must be present. In turn, it must contain Mn to obtain high N_{max} and Mg to obtain high V_{max} . Therefore, the 4 selected media were: WP + YE 4 g/L + WH 7 g/L + Mn + Mg; WP + YE 8 g/L + WH 7 g/L + Mn + Mg; WP + YE 8 g/L + WH 14 g/L + Mn + Mg and WP + YE 4 g/L + WH 14 g/L + Mn + Mg (Table 2).

N_{max} and V_{max} were higher in the 4 selected media than N_{max} ($P < 0.001$) and V_{max} ($P < 0.001$) obtained in the medium without supplementation (WP 60 g/L).

Likewise, V_{max} of the 4 selected media were the same, but, N_{max} were different between them, obtaining the highest amount of biomass in the medium WP 60 g/L + YE 4g/L + WH 7 g/L + Mg + Mn (Table 2). The growth curve obtained in this medium had a lower N_{max} and V_{max} than the curve obtained in MRS, but both had the same T_{vmax} and Lag fase (Table 2).

L. salivarius DSPV008P

The highest amount of biomass (N_{max}) was obtained when YE and WH were at 4 g/L and 8 g/L ($P < 0.001$), and 7 g/L and 14 g/L ($P = 0.047$), respectively, and Mg was at 0.02 g/L ($P = 0.008$) in the culture medium. In turn, the presence of Mn produced a negative effect on N_{max} ($P < 0.001$), so this supplement should be absent (Table 3).

Supplements that increased the kinetic parameter V_{max} were the presence of YE and WH at 4 g/L y 8 g/L ($P < 0.001$) and 7 g/L and 14 g/L ($P = 0.016$), respectively. Mg ($P = 0.073$) and Mn ($P = 0.785$) did not produce any effect on V_{max} (Table 3). Thus, it follows that the 4 media coincided with the highest levels of N_{max} and V_{max} were: WP + YE 4 g/L + WH 14 g/L + Mg; WP + YE 8 g/L + WH 7 g/L + Mg; WP + YE 8 g/L + WH 14 g/L + Mg and WP + YE 4 g/L + WH 7 g/L + Mg (Table 4).

Even though the factors that benefited the growth of *L. salivarius* DSPV008P were determined, the selected media did not allow an adequate growth. The selected media generated an increase in biomass compared to the basal medium WP 60 g/L, but the growth was well below the growth in MRS. Therefore, none of the selected media was suitable for the development of this strain (Table 4).

L. salivarius DSPV011P

The highest amount of biomass (N_{max}) was obtained when YE and Mn were at 4 g/L and 8 g/L ($P < 0.001$) and 0,003 g/L ($P < 0.001$), respectively in the culture medium. On the other hand, Mg and WH did not affect the growth of the strain ($P = 0.383$ and $P = 0.508$, respectively) (Table 5).

Supplement that increased the kinetic parameter V_{max} was YE in its 2 concentrations: 4 g/L and 8 g/L ($P < 0.001$). Further, Mg, Mn and WH did not affect V_{max} ($P = 0.199$, $P = 0.638$ and $P = 0.146$, respectively) (Table 5). Therefore, the 2 selected media were: WP 60 g/L + YE 4 g/L + Mn and WP 60 g/L + YE 8 g/L + Mn, but the latter was the one that produced the largest amount of biomass at the highest speed (Table 6).

Furthermore, N_{max} of the selected medium (WP 60 g/L + YE 8 g/L + Mn) was equal to N_{max} in MRS. In the same way, all the other kinetic parameters evaluated were similar in the selected medium and MRS, generating similar growth curves (Table 6).

Tabla 1. *Lactobacillus salivarius* DSPV002 kinetic parameters for factors: yeast extract, whey hydrolyzate, $MgSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$ in its different concentrations. V_{max} : the

maximum specific growth rate; Nmax: the maximum number of bacteria reached. Different letters mean significant differences ($P < 0.05$) among different concentrations of each factor for each kinetic parameter.

Factors	Concentration	Nmax (Mean \pm SD)	Vmax (Mean \pm SD)
Yeast extract	0 g/L	0.30 \pm 0.120 ^b	1.29 \pm 1.250 ^b
	4 g/L	0.77 \pm 0.246 ^a	2.34 \pm 0.829 ^a
	8 g/L	0.83 \pm 0.212 ^a	2.34 \pm 0.774 ^a
MgSO ₄ .7H ₂ O	0	0.63 \pm 0.317 ^a	1.86 \pm 1.143 ^b
	0.02	0.64 \pm 0.330 ^a	2.12 \pm 1.022 ^a
	0 g/L	0.54 \pm 0.307 ^b	2.25 \pm 1.158 ^b
Whey hydrolyzate	7 g/L	0.69 \pm 0.345 ^a	2.90 \pm 0.995 ^a
	14 g/L	0.68 \pm 0.287 ^a	2.82 \pm 0.889 ^a
	0	0.59 \pm 0.281 ^b	1.98 \pm 1.107 ^a
MnSO ₄ .H ₂ O	0	0.59 \pm 0.281 ^b	1.98 \pm 1.107 ^a
	0.003	0.68 \pm 0.358 ^a	2.00 \pm 1.079 ^a

Tabla 2. *Lactobacillus salivarius* DSPV002 kinetic parameters for selected media, WP (whey permeate) without supplementation and positive control (MRS). YE: yeast extract; WH: whey hydrolysate; Mn: MnSO₄.H₂O; Mg: MgSO₄.7H₂O; Vmax: the maximum specific growth rate; TVmax: the time it takes the bacteria to reach the maximum growth rate; Fase Lag: the lag time or Lag phase; Nmax: the maximum number of bacteria reached. Different letters mean significant differences ($P < 0.05$) among the selected media.

Selecte d Media	Nmax (DO)	Vmax	TVmax (Hours)	Fase Lag (Hours)
WP 60 g/l	0.20 \pm 0.010 ^f	0.44 \pm 0.038 ^c	02:47 \pm 00:47 ^a	01:39 \pm 01:42 ^a
WP 60 g/l + YE 4g/l + WH 7 g/l + Mg + Mn	1.16 \pm 0.550 ^b	2.91 \pm 0.867 ^b	05:45 \pm 00:51 ^a	03:15 \pm 00:06 ^a
WP 60 g/l + YE 4g/l + WH 14 g/l + Mg + Mn	0.59 \pm 0.115 ^c	2.25 \pm 1.091 ^b	06:34 \pm 00:51 ^a	05:06 \pm 03:05 ^a
WP 60 g/l + YE 8g/l + WH 7 g/l + Mg + Mn	1.04 \pm 0.080 ^c	2.23 \pm 1.127 ^b	04:45 \pm 00:34 ^a	02:40 \pm 00:06 ^a
MRS	1.38 \pm 0.080 ^a	6.03 \pm 0.264 ^a	05:15 \pm 01:00 ^a	02:39 \pm 00:45 ^a

Biomass production and economic cost of selected media

L. salivarius DSPV002P viable cells count obtained in WP medium was lower (7.05 log CFU/mL) than in the selected medium (WP 60 g/l + YE 4g/l + WH 7 g/l + Mg + Mn, 8.31 log CFU/mL). However, growth in the latter medium was lower than in MRS medium (8.68 log CFU/mL) ($P < 0.001$). Nevertheless, biomass production cost in MRS (\$USD 120.55/1000L) was 20 times higher than in selected medium (\$USD 5.75/1000L).

Tabla 3. *Lactobacillus salivarius* DSPV008 kinetic parameters for factors: yeast extract, whey hydrolyzate, MgSO₄.7H₂O and

MnSO₄.H₂O in its different concentrations. Vmax: the maximum specific growth rate; Nmax: the maximum number of bacteria reached. Different letters mean significant differences ($P < 0.05$) among different concentrations of each factor for each kinetic parameter.

Factors	Concentration	Nmax (Mean \pm SD)	Vmax (Mean \pm SD)
Yeast extract	0 g/L	0.15 \pm 0.044 ^b	0.53 \pm 0.309 ^b
	4 g/L	0.18 \pm 0.032 ^a	0.95 \pm 0.246 ^a
	8 g/L	0.20 \pm 0.023 ^a	1.12 \pm 0.283 ^a
MgSO ₄ .7H ₂ O	0	0.17 \pm 0.040 ^b	0.87 \pm 0.364 ^a
	0.02	0.18 \pm 0.049 ^a	0.86 \pm 0.383 ^a
	0 g/L	0.17 \pm 0.033 ^b	0.79 \pm 0.298 ^b
Whey hydrolyzate	7 g/L	0.18 \pm 0.048 ^a	0.86 \pm 0.397 ^a
	14 g/L	0.18 \pm 0.051 ^a	0.95 \pm 0.410 ^a
	0	0.20 \pm 0.045 ^a	0.82 \pm 0.345 ^a
MnSO ₄ .H ₂ O	0	0.20 \pm 0.045 ^a	0.82 \pm 0.345 ^a
	0.003	0.16 \pm 0.036 ^b	0.91 \pm 0.404 ^a

Tabla 4. *Lactobacillus salivarius* DSPV008 kinetic parameters for selected media, WP (whey permeate) without supplementation and positive control (MRS). YE: yeast extract; WH: whey hydrolysate; Mn: MnSO₄.H₂O; Mg: MgSO₄.7H₂O; Vmax: the maximum specific growth rate; TVmax: the time it takes the bacteria to reach the maximum growth rate; Fase Lag: the lag time or Lag phase; Nmax: the maximum number of bacteria reached. Different letters mean significant differences ($P < 0.05$) among the selected media.

Selecte d Media	Nmax (DO)	Vmax	TVmax (Hours)	Fase Lag (Hours)
WP 60 g/l	0.13 \pm 0.020 ^e	0.43 \pm 0.130 ^e	02:45 \pm 00:15 ^a	01:23 \pm 00:24 ^c
WP 60 g/l + YE 4g/l + WH 7 g/l + Mg	0.26 \pm 0.020 ^b	0.87 \pm 0.150 ^d	01:25 \pm 00:31 ^a	00:19 \pm 00:19 ^a
WP 60 g/l + YE 4g/l + WH 14 g/l + Mg	0.25 \pm 0.010 ^b	1.12 \pm 0.140 ^c	01:55 \pm 00:54 ^a	00:55 \pm 00:01 ^{abc}
WP 60 g/l + YE 8g/l + WH 7 g/l + Mg	0.26 \pm 0.010 ^b	1.12 \pm 0.120 ^c	01:55 \pm 00:15 ^a	00:55 \pm 00:15 ^{abc}
MRS	0.27 \pm 0.020 ^b	1.48 \pm 0.100 ^b	01:25 \pm 00:17 ^a	00:36 \pm 00:05 ^{ab}

On the other hand, *L. salivarius* DSPV011P biomass obtained in WP medium was lower (7.05 log CFU/mL) than biomass obtained in the selected medium (WP + YE 8 g/L + Mn, 8.93 log CFU/mL) and in MRS (8.95 log CFU/mL) ($P < 0.001$). Further, production costs of *L. salivarius* DSPV011P biomass would be 12 times less in the selected media (\$USD 9.88/1000L) than in MRS (\$USD 120.55/1000L).

Since *L. salivarius* DSPV 011P was the only strain capable of generating an Nmax equal to MRS, biomass production in the selected medium was further evaluated in a laboratory scale bioreactor. Under these

conditions, the biomass production was 9.22 log (CFU/mL), increasing 0.29 log (CFU/mL) more than in the test tube.

L. salivarius DSPV 008P viable cell count analysis was not performed because this strain did not grow adequately in any of the formulated media.

Tabla 5. *Lactobacillus salivarius* DSPV011 kinetic parameters for factors: yeast extract, whey hydrolyzate, MgSO₄.7H₂O and MnSO₄.H₂O in its different concentrations. Vmax: the maximum specific growth rate; Nmax: the maximum number of bacteria reached. Different letters mean significant differences (P <0.05) among different concentrations of each factor for each kinetic parameter.

Factors	Concentration	Nmax (Mean ± SD)	Vmax (Mean ± SD)
Yeast extract	0 g/L	0.27±0.139 ^b	0.87±0.567 ^b
	4 g/L	0.90±0.171 ^a	2.39±0.958 ^a
	8 g/L	0.87±0.177 ^a	3.09±1.219 ^a
MgSO ₄ .7H ₂ O	0	0.68±0.354 ^a	2.167±1.306 ^a
	0.02	0.69±0.297 ^a	2.064±1.337 ^a
Whey hydrolyzate	0 g/L	0.68±0.421 ^a	2.20±1.145 ^a
	7 g/L	0.65±0.285 ^a	1.93±1.278 ^a
	14 g/L	0.73±0.252 ^a	2.22±1.259 ^a
MnSO ₄ .H ₂ O	0	0.64±0.093 ^b	2.15±1.266 ^a
	0.003	0.73±0.111 ^a	2.08±1.394 ^a

Tabla 6. *Lactobacillus salivarius* DSPV011 kinetic parameters for selected media, WP (whey permeate) without supplementation and positive control (MRS). YE: yeast extract; WH: whey hydrolysate; Mn: MnSO₄.H₂O; Mg: MgSO₄.7H₂O; Vmax: the maximum specific growth rate; TVmax: the time it takes the bacteria to reach the maximum growth rate; Fase Lag: the lag time or Lag phase; Nmax: the maximum number of bacteria reached. Different letters mean significant differences (P <0.05) among the selected media.

Selecte d Media	Nmax (DO)	Vmax	TVmax (Hours)	Fase Lag (Hours)
WP 60 g/l	0.22±0.062 ^c	0.50±0.033 ^c	02:15±00:34 ^a	01:06±00:49 ^a
WP 60 g/l + YE 4g/l + Mn	1.05±0.152 ^b	3.23±0.333 ^b	03:34±00:17 ^a	01:21±00:14 ^a
WP 60 g/l + YE 8g/l + Mn	1.29±0.132 ^a	4.46±0.121 ^a	02:34±00:17 ^a	00:52±00:13 ^a
MRS	1.34±0.132 ^a	4.78±0.184 ^a	03:55±00:21 ^a	01:36±00:14 ^a

Discussion

For the formulation of an industrial-culture medium is necessary that it has all the nutritional requirements to

support an appropriate microorganism growth. These additives have to be abundant in the market and of low production cost (León de la O *et al.*, 2013; Vázquez *et al.*, 2009). In this study, different formulations of culture media were evaluated taking into account the kinetic characteristics of growth of three strains of *L. salivarius* from avian origin, which manifested different nutritional requirements, different growth times and reached different final counts.

The applied statistical methodology was very useful to identify the factors effects on the biomass production and with these data the most appropriate medium was made. These media produced different results according to the strain: an optimal growth was obtained for *L. salivarius* DSPV011P; a high growth was obtained for *L. salivarius* DSPV002P, but not as high as that obtained in MRS medium; and low growth was obtained for *L. salivarius* DSPV008P. Different studies have reported that the inclusion of YE or protein lysate, magnesium sulfate, manganese sulfate and vitamins improve the production of lactic acid in cheese whey used as growth media (Cui *et al.*, 2012; Kwon *et al.*, 2000; Vázquez *et al.*, 2009). Likewise, differences in the nutritional requirements of microorganisms can promote or suppress the definitive genetic properties of *Lactobacillus* spp. through epigenetic reprogramming (Shenderov, 2014). In addition, there is a high heterogeneity in the metabolic properties among species and bacterial strains (Mikelsaar *et al.*, 2015). However, most LAB require an exogenous nitrogen source of amino acids or peptides to support cell growth (Manca de Nadra, 2007).

Among all the factors evaluated, the addition of YE to the base medium (WP) improved the growth of two of the three strains under study. Microorganisms with complex nutritional requirements may need small amounts of essential nutrients. Such nutrients, called growth factors (Amrane y Prigent, 1998), include purines and pyrimidines (necessary for the synthesis of nucleic acids), amino acids (necessary for the synthesis of proteins) and vitamins (necessary as coenzymes and functional groups of certain enzymes). Growth factors are not directly metabolized as carbon or energy sources; rather they are assimilated by the cells to fulfill their specific role in the metabolism (Manca de Nadra, 2007). The use of YE is advantageous over other possible nitrogenous sources, since it also provides vitamins and bases of nucleic acids essential for the production of purified proteins (Amrane y Prigent, 1998).

It has been described that some probiotic strains improve the yield of biomass production when hydrolysates are added to the formulated medium (Dabrowska *et al.*, 2017; Kareba *et al.*, 2018). Nonetheless, growth kinetics may vary with the presence and/ or concentration of WH depending on the strain. Each strain can have different peptide transport systems which can carry free amino acids, di- and tri-peptides and oligopeptides of up to six amino acid residues. Therefore, the growth capacity will depend on the degree of hydrolysis achieved in the WH

and the pathway for the transport of amino acids of each strain (Dabrowska *et al.*, 2017). In this study, WH improved the microbial development of *L. salivarius* DSPV002P.

The strains also differed in the use of mineral salts for the development of their biomass. The mineral salts used in this work, Mn and Mg, are of utmost importance for cell growth since they function as essential co-factors for the enzymes in the primary metabolism of LAB sugars (Madigan *et al.*, 2004). In addition, both metal ions play a central role in the production of NAD and ATP and, therefore, are essential for many cellular functions (Vinderola *et al.*, 2000). Such is the case of *L. salivarius* DSPV002P, since it required the addition of both salts minerals. However, *L. salivarius* DSPV011P had its highest yields in the presence of Mn and without the addition of Mg. This may be due these two divalent metal ions have very similar chemical properties and Mg ions can be exchanged for Mn ions at the metal binding sites of many proteins (Lew *et al.*, 2012). However, the Mn requirement has a certain degree of specificity and it cannot be completely replaced by other metals (Raccach, 1985).

Studying the kinetic parameters of the growth of microorganisms allows to optimize and standardize the fermentation process both at the laboratory level and at the industrial scale (Kareba *et al.*, 2018; Zapata *et al.*, 2005). In this sense, although the value of Nmax is considered the most important at the level of biomass yield, the other parameters play an important role in the overall evaluation of the process. It is known that the lag phase is the time in which a culture passes from the adaptation phase (Lag) to the logarithmic growth phase (Log) in any kinetic growth study (León de la O *et al.*, 2013). If this can be shortened by optimizing the culture medium, it will allow the inoculated strain to dominate the fermentation process, preventing possible biological contaminants. In this way, it can be inferred that this microorganism could adapt better to the supplemented medium than to the commercial medium, which implies an advantage for the large-scale fermentation technological process, reducing costs. The maximum growth rate was lower in the medium formulated for *L. salivarius* DSPV002P and *L. salivarius* DSPV008P than the commercial medium, but there was no difference for the case of *L. salivarius* DSPV011P. However, it is known that the specific rate of growth can improve when the growth media are optimized as was described by Zacharof *et al.* (2009), who reported increases of up to 300% in the different strains studied. Previous studies have shown that other mineral salts like K_2HPO_4 , NaAc and $(NH_4)_2SO_4$ or different concentrations of the salts evaluated (Cui *et al.*, 2012; Macedo *et al.*, 2002; Trigueros *et al.*, 2016) could optimize the biomass of the strains, because the addition of micronutrients can enhance their role in cell growth.

In relation to the biomass obtained, the results showed that only the strain *L. salivarius* DSPV011P grew in the formulated medium in a similar way to commercial

medium. In the case of the evaluation of the costs of all the formulations, they turned out to be significantly cheaper than the commercial medium. In particular, the medium formulated for *L. salivarius* DSPV011P cost approximately 12 times less than the commercial medium. As mentioned above, the use of low-cost growth media to obtain probiotic biomass is of great interest at an industrial level to reduce costs and extend its incorporation to feed (León de la O *et al.*, 2013; Vázquez *et al.*, 2009). In this sense, by-products such cheese whey permeate constitute an alternative for the production of probiotic bacteria (Lavari *et al.*, 2015) destined to slaughter animals.

Although the effect of supplements added to the culture medium on kinetic parameters are strain dependent, *L. salivarius* DSPV011P is the strain with the best technological characteristics, capable of growing in a medium based on a by-product of the dairy industry supplemented with YE and Mn and at a cost 12 times lower than in MRS. However, future studies must be carried out to determine that the physiological properties of this strain are not modified by growth in this formulated medium. The development of a low-cost culture medium for the generation of potential probiotic biomass, would make possible the transfer of *L. salivarius* DSPV011P and the medium developed for its production on an industrial scale for its application in broilers farms.

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