Research Article

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Different combinations of salts affect the growth and bacteriocin production by *Lactobacillus salivarius* CRL 1328

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Abstract

BACKGROUND: The culture medium for optimal growth of vaginal *Lactobacillus salivarius* CRL 1328 is different from that for optimal bacteriocin production. To simultaneously obtain high amount of biomass and bacteriocin of this microorganism, the effects of different basal culture media and salts on both responses were evaluated. The study was performed by using a complete factorial experimental design 2^6, with central points. Sixty-four different growth media, which resulted from the combinations of two basal culture media and two concentrations of five salts (ammonium citrate, sodium acetate, $MgSO_4$, $MnSO_4$, and K_2HPO_4) were assayed.

RESULTS: Only the addition of $MnSO_4$ to each culture medium significantly stimulated the growth of *L. salivarius*. The presence of sodium acetate or $MgSO_4$ stimulated the bacteriocin production, while $MnSO_4$ and K_2HPO_4 exerted an inhibitory effect. However, the simultaneous addition of $MnSO_4$ and sodium acetate to both basal culture media allowed high bacteriocin levels to be reached, attenuating the inhibitory effect of Mn^{2+}.

CONCLUSIONS: The application of a complete experimental design contributed to simultaneous optimization of the biomass and bacteriocin production of *L. salivarius* CRL 1328. The results obtained are potentially applicable to the technological production of probiotic bacteria and antagonistic substance to be included in a probiotic pharmaceutical product.

Supporting information may be found in the online version of this article.

Keywords: vaginal *Lactobacillus*; bacteriocin; salt combinations; complete factorial design.

INTRODUCTION

Lactic acid bacteria (LAB) are a group of microorganisms nutritionally exigent. They need a wide range of nutrients to grow and synthesize metabolic products, some nutritional requirements usually being strain specific.[1] De Man-Rogosa-Sharpe (MRS) and yeast autolysate-peptone-tryptone-tween 80-glucose (LAPTg) are standard culture media commonly used to support the growth of lactobacilli.[2,3] These media contain carbon and energy sources (carbohydrates, e.g. glucose), complex nitrogen sources (yeast extract, meat extract, tryptone and peptone) and supplements derived from oleic acid (Tween 80). MRS also includes inorganic and organic salts that have shown a stimulating effect or are essential for the growth of most of the species of this genus. Different components of culture media strongly affect the growth and bacteriocin production of several microorganisms that are mainly considered for food applications.[4–10]

Lactobacillus salivarius subsp. *salivarius* CRL 1328, isolated from human vagina, is a microbial candidate to be included in a probiotic formula to prevent or treat urogenital infections in women.[11] This microorganism evidences some beneficial and technological characteristics, demonstrated previously through '*in vitro*' assays, as follows: inhibits the growth of different urogenital pathogens, such as *Enterococcus faecalis, Enterococcus faecium* and *Neisseria gonorrhoeae* by a bacteriocin identified as salivaricin CRL 1328,[12,13] inhibits the growth of *Escherichia coli, Klebsiella* sp., *Gardnerella vaginalis, Staphylococcus aureus* and *Streptococcus agalactiae* by the low pH produced;[14] is able of auto-aggregate and co-aggregate with vaginal *Candida* sp.;[15] shows a good performance and robustness during storage after freeze or freeze-drying.[16,17]

Determination of the optimum conditions to obtain the highest amount of viable microorganisms, as well as to produce bacteriocins, can be performed by using the classical 'one-factor at a time' methodology or by applying factorial experimental designs.[18] A complete factorial design is an organized and efficient method to explore all the possible combinations (treatments) of the factors (variables) under study and their levels. Factorial experiments have been used to test the effects of several physico-chemical factors influencing cell growth and bacteriocin production, mainly of microorganisms for food applications.[6,7,9,19,20]

Through the use of a complete factorial design and statistical analysis, the optimal values of initial pH and temperature for the growth of *L. salivarius* CRL 1328 was determined, being coincident

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with those for bacteriocin production.[21] However, optimal culture media were different for each response: the growth was higher in MRS broth than in LAPTg broth, and the maximal bacteriocin production was recorded in LAPTg. Then, the hypothesis that some salts and mineral ions supplementing MRS are able to stimulate the growth of *L. salivarius* CRL 1328 but not to increase the salivaricin production was built.

The objective of the present work was to evaluate the combined effects of different salts on the growth and bacteriocin production of *L. salivarius* CRL 1328. The study was performed by using a complete 2^6 factorial experimental design. In this way, an improvement in the design of an optimal culture medium for efficient production of both responses is demonstrated.

Table 1. Factors and levels used for the complete factorial experimental design applied for the evaluation of the effects of different salts on the growth and bacteriocin production of *L. salivarius* CRL 1328

	Level of factors		
Factors	Low	Central Point	High
Basal culture medium	CNS-MRS[a]	–	LAPTg
Ammonium citrate (%)	0	0.1	0.2
Sodium acetate (%)	0	0.25	0.5
$MgSO_4$ (%)	0	0.005	0.01
$MnSO_4$ (%)	0	0.0025	0.005
K_2HPO_4 (%)	0	0.1	0.2

[a] Carbon and nitrogen sources of MRS.

MATERIALS AND METHODS

Bacterial strains and culture media

Bacteriocin producing *L. salivarius* CRL 1328 (from the CERELA Culture Collection, San Miguel de Tucumán, Tucumán, Argentina) and *Enterococcus faecalis* MP97 (indicator strain) were isolated from vaginal samples and stored in a milk-yeast extract (13% non-fat milk, 0.5% yeast extract and 1% glucose) at −70 °C.[11,12]

Before the growth experiments, *L. salivarius* was propagated three times in MRS (0.5% yeast extract, 1% meat peptone, 1% meat extract, 2% glucose, 0.2% ammonium citrate, 0.5% sodium acetate, 0.01% $MgSO_4$, 0.005% $MnSO_4$, 0.2% K_2HPO_4, 0.1% Tween 80; pH 6.5)[2] at 37 °C. The third culture of 12 h of incubation was centrifuged (10 min, 6000g), washed with a saline solution (0.85% NaCl), and the cell pellet was then resuspended in the same solution to reach a final optical density of 1.4 at 540 nm ($OD_{540\ nm}$) to inoculate the different culture media to be assayed. Before the inhibition assays, *E. faecalis* was propagated twice in LAPTg (1% yeast extract, 1.5% meat peptone, 1% tryptone, 1% glucose, 0.1% Tween 80; pH 6.5)[3] broth at 37 °C for 12 h; the $OD_{540\ nm}$ of the final culture was adjusted to 0.6.

The culture media evaluated in this work were prepared from concentrated solutions of the different components: 5X LAPTg broth (5% yeast extract, 7.5% meat peptone, 5% tryptone, 5% glucose and 0.5% Tween 80), 5X carbon and nitrogen sources from MRS broth (CNS-MRS: 2.5% yeast extract, 5% meat peptone, 5% meat extract, 10% glucose and 0.5% Tween 80), 2% ammonium citrate, 5% sodium acetate, 0.1% $MgSO_4.7H_2O$, 0.05% $MnSO_4.H_2O$, 2% K_2HPO_4. Sufficient volume of concentrated solutions was added for each one of the growth media assayed, to reach the final concentration of each specified component in the different experimental designs. The initial pH of the growth media was adjusted to 6.5, the pH determined previously as the optimal for the growth and bacteriocin synthesis of *L. salivarius* CRL 1328,[21] and the media autoclaved at 121 °C for 20 min.

Growth conditions

L. salivarius CRL 1328 was inoculated (2% v/v) into 5 mL of each of the growth media assayed, and aliquots were distributed on micro test tubes (a tube for each time point), and then incubated at a temperature of 37 °C, without agitation. Samples were taken at specific time intervals (0, 6, 9, 12 and 24 h) and placed in 96-well microplates. $OD_{540\ nm}$ measurements were performed in a microplate reader (Spectrophotometer Molecular Devices, VERSA max, USA). The inhibitory activity of the supernatant fluid of *L. salivarius* CRL 1328 was determined by an agar diffusion method, as described previously.[21] The bacteriocin titre was expressed as logarithm of arbitrary units per milliliter (log (AU mL^{-1})).

Factorial experimental design

The combined effects of salts were assayed in a 2^6 complete factorial experimental design. The six factors considered were: basal culture medium (BCM) at two levels [LAPTg or carbon and nitrogen sources of MRS (CNS-MRS), which were employed to test the resulting salt combinations] and the five salts present in standard MRS medium, each one at two different concentrations (Table 1). To evaluate the effect of the BCM assayed (qualitative variable) statistically, CNS-MRS was arbitrarily considered as 'low level' and LAPTg as 'high level'. In each BCM, a central point was also assayed, which represents half of the concentrations of each one of the salts tested. The combination that represented the central point was assayed twice. A total of 68 growth curves were analyzed.

Statistical data analysis

The Experimental Design Module of the Statistica 6.0 software package (Statistica for Windows computer program manual; StatSoft Inc.) was used for the elaboration of experimental designs and analysis of data. The statistical analysis was performed by considering the maximum values of $OD_{540\ nm}$ and log (AU mL^{-1}) obtained at up to 12 h of growth, in each of the growth media tested. The logarithmic transformation of bacteriocin titres was used to reduce the variation due to the wide range of activity values obtained. The response surface methodology was applied to analyze the role of the independent variable interactions and to predict the levels of factors required for the optimum conditions for biomass and bacteriocin production (Statistica 6.0 software).

RESULTS

The kinetics of growth ($OD_{540\ nm}$) and bacteriocin production [log (AU mL^{-1})] of *L. salivarius* CRL 1328 under varying culture conditions are summarized in Figs 1 and 2. The results demonstrate that the responses of interest depended on the different culture conditions used, as detailed below. On the other hand, Table 2 summarizes the results of the statistical evaluation of $OD_{540\ nm}$ and log (AU mL^{-1}) variations of *L. salivarius* CRL 1328. The different factors considered (BCM and salts) were show to exert different effects on growth and bacteriocin production, as explained later.

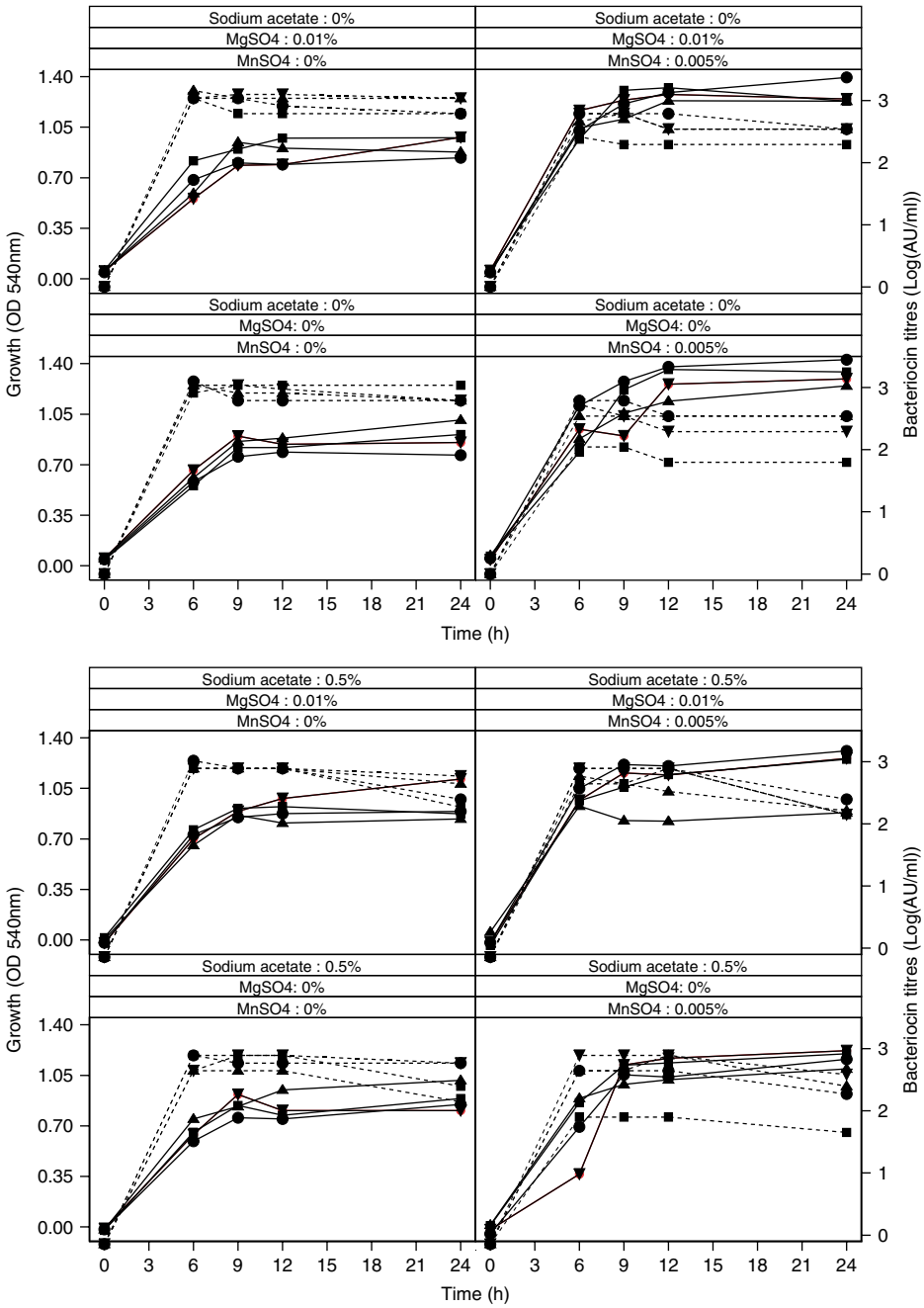

Figure 1. Kinetics of growth (solid lines) and bacteriocin production (dashed lines) of *L. salivarius* CRL 1328 cultured in LAPTg broth, in the presence of different salt combinations. (▼) 0% ammonium citrate, 0% K_2HPO_4; (▲) 0% ammonium citrate, 0.2% K_2HPO_4; (●) 0.2% ammonium citrate, 0% K_2HPO_4, (■) 0.2% ammonium citrate, 0.2% K_2HPO_4.

Effects of nutrients on the growth of *L. salivarius* CRL 1328

The BCM did not significantly influence the growth of *L. salivarius* CRL 1328. Thus, the maximum $OD_{540\ nm}$ values obtained were similar in both LAPTg and CNS-MRS, for the same salt combination assayed. Among the different salts tested, only $MnSO_4$ significantly stimulated the growth of *L. salivarius* (magnitude of the effect $=$ 0.32 $OD_{540\ nm}$ units; $P < 0.05$). Thus, the addition of 0.005% $MnSO_4$ to BCM was shown to enable the highest cell concentration recorded in the experimental design used ($OD_{540\ nm} = 1.24 \pm 0.10$, at 6–12 h of culture). These $OD_{540\ nm}$ values were similar to those obtained in standard MRS (1.30 at 12 h of culture). However, in the growth media without $MnSO_4$, the biomass values obtained were significantly lower ($OD_{540\ nm} = 0.91 \pm 0.10$) (Figs 1 and 2; Table 2).

Many factor interaction effects were observed that showed lower significance (lower magnitude) than the positive effect produced only by $MnSO_4$ (Table 2). The response surface plot (Fig. 3) shows the significant interaction effects of $MnSO_4$ and ammonium citrate or sodium acetate (in LAPTg) on the growth and bacteriocin production. The positive effect of $MnSO_4$ on the $OD_{540\ nm}$ values was higher in the presence of 0.2% ammonium citrate (positive interaction effect), but lower in the presence of sodium acetate (negative interaction effect).

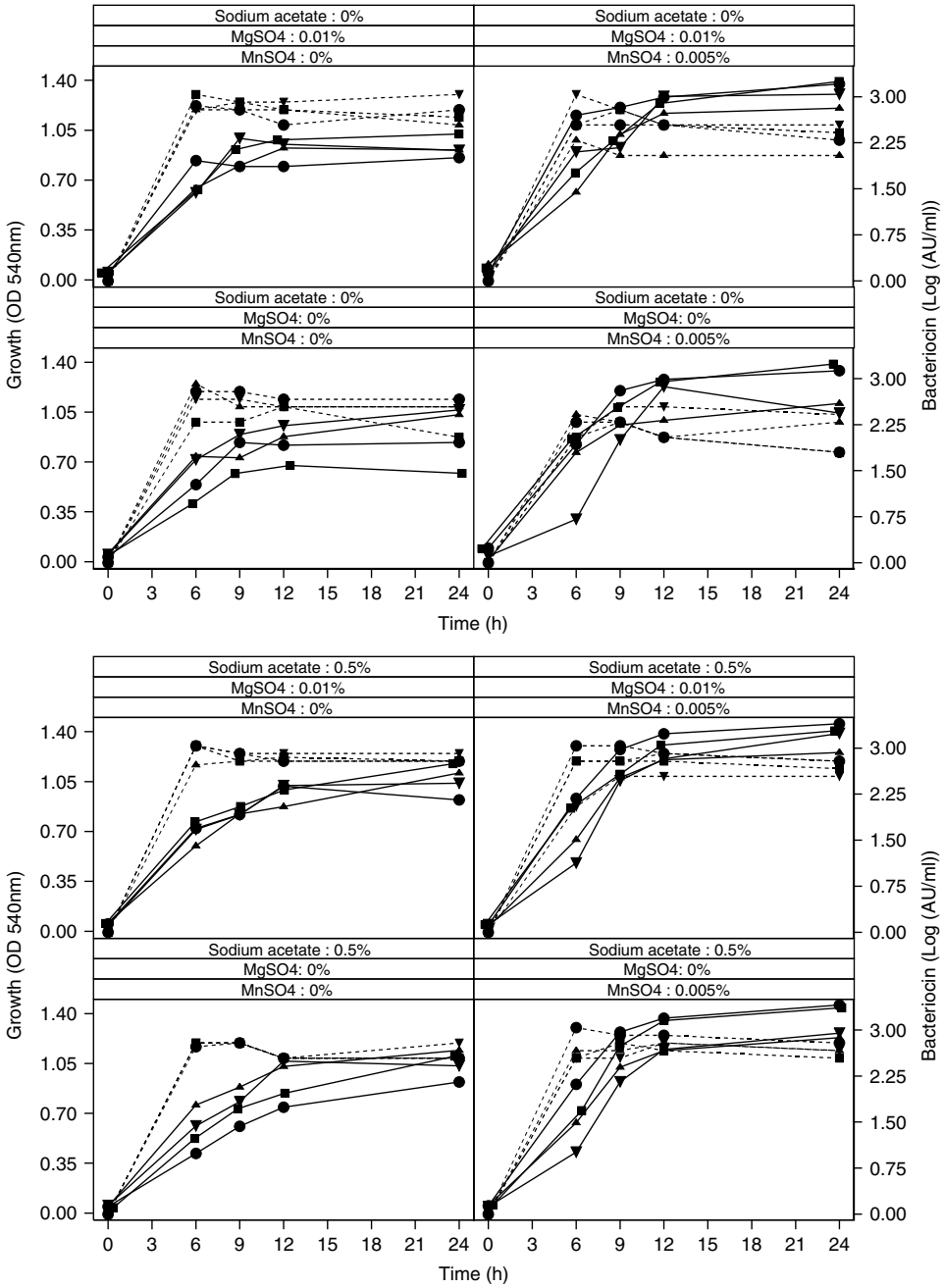

Figure 2. Kinetics of growth (solid lines) and bacteriocin production (dashed lines) of *L. salivarius* CRL 1328 cultured in CNS-MRS broth, in the presence of different salt combinations. (▼) 0% ammonium citrate, 0% K_2HPO_4; (▲) 0% ammonium citrate, 0.2% K_2HPO_4; (●) 0.2% ammonium citrate, 0% K_2HPO_4, (■) 0.2% ammonium citrate, 0.2% K_2HPO_4.

Referring to the growth rates (Tables 4 and 5, supporting information), the factors evaluated did not show a statistically significant effect on the calculated values (Table 3).

Effects of nutrients on bacteriocin production

The results of the statistical analysis of the experimental design applied demonstrated that salivaricin CRL 1328 production was significantly affected by most of the factors evaluated (BCM and four salts), in contrast to the results described earlier for the growth of the bacteriocin-producer microorganism (Table 2). Only ammonium citrate did not show a statistically significant influence on the bacteriocin levels.

The positive effect of BCM on bacteriocin production [magnitude of 0.13 log (AU mL^{-1}) units] indicates that the response was higher when employing the carbon and nitrogen sources of LAPTg medium (high level of BCM factor) than those of MRS medium (low level of BCM factor), for the same salt combination assayed (Table 2).

LAPTg medium without salts (standard LAPTg) was shown to contain enough energy sources to support high bacteriocin production [log (AU mL^{-1}) = 3.04 at 6 h]. The addition of different salt combinations allowed similar maximum bacteriocin titres to be obtained in most of cases evaluated. However, in some of the growth media containing $MnSO_4$ and/or K_2HPO_4, the bacteriocin

Table 2. Statistical evaluation of the main and interactions effects produced by the different basal culture media and salts assayed on the growth and bacteriocin production of vaginal *L. salivarius* CRL 1328

	Statistical analysis for maximal OD values			Statistical analysis for maximal log (AU mL^{-1}) values		
Factor	Effect ($OD_{540\ nm}$ units)[a]	*t*-value	*P*-value*	Effect (log AU mL^{-1} units)[a]	*t*-value	*P*-value*
Mean/Intercept	2.628	35.73	0.00	5.98	31.92	0.00
Basal culture medium (BCM)[b]	−0.02	−1.30	0.20	0.13	2.73	0.01
Ammonium citrate (AC)	0.01	0.70	0.49	−0.04	−0.76	0.45
Sodium acetate (SA)	0.01	0.68	0.50	0.14	3.02	0.00
$MgSO_4$	0.03	1.76	0.08	0.14	3.02	0.00
$MnSO_4$	0.33	17.63	0.00	−0.24	−5.07	0.00
K_2HPO_4	−0.02	−1.33	0.19	−0.10	−2.20	0.03
BCM × AC	0.01	0.39	0.70	−0.07	−1.46	0.15
AC × SA	0.01	0.40	0.69	0.05	1.09	0.28
AC × $MgSO_4$	0.04	1.93	0.06	0.07	1.58	0.12
AC × $MnSO_4$	0.09	4.78	0.00	−0.06	−1.33	0.19
AC × K_2HPO_4	0.04	2.21	0.03	−0.04	−0.92	0.36
BCM × SA	−0.03	−1.66	0.10	−0.04	−0.80	0.42
SA × $MgSO_4$	0.00	−0.04	0.96	−0.02	−0.39	0.70
SA × $MnSO_4$	−0.06	−3.19	0.00	0.13	2.77	0.01
SA × K_2HPO_4	−0.01	−0.67	0.50	0.01	0.23	0.82
BCM × $MgSO_4$	−0.01	−0.74	0.46	0.00	0.02	0.98
$MgSO_4$ × $MnSO_4$	−0.02	−1.16	0.25	0.02	0.47	0.64
$MgSO_4$ × K_2HPO_4	0.00	−0.19	0.85	0.04	0.88	0.38
BCM × $MnSO_4$	−0.01	−0.39	0.70	−0.07	−1.41	0.16
$MnSO_4$ × K_2HPO_4	−0.03	−1.54	0.13	−0.07	−1.46	0.15
BCM × K_2HPO_4	0.01	0.75	0.45	−0.06	−1.17	0.25

[a] Magnitude of the effect of each factor: For the BCM, this value indicates the difference of $OD_{540\ nm}$ or log (AU mL^{-1}) produced by the use of LAPTg and CNS-MRS; for the salts, this magnitude indicates the difference of $OD_{540\ nm}$ or log (AU mL^{-1}) obtained when added to the growth medium. Positive values of effects mean stimulation of biomass or bacteriocin production. Negative values mean inhibition of the responses of interest.
[b] Basal culture media: LAPTg and CNS-MRS.
* $P < 0.05$ indicates statistically significant effect.

titres were lower than those observed in standard LAPTg (Fig. 1). In CNS-MRS, the bacteriocin titres were low [maximum log (AU mL^{-1}) = 2.66 at 6 h of culture] and the values increased mainly because of the addition of sodium acetate and/or $MgSO_4$ (Fig. 2). In CNS-MRS supplemented with the five salts assayed (standard MRS), the higher bacteriocin levels were around 2.8 log (AU mL^{-1}) at 6–12 h of incubation.

Table 2 shows that the presence of sodium acetate or $MgSO_4$ positively affected bacteriocin production. However, $MnSO_4$ and K_2HPO_4 exerted a significant inhibitory effect on the production of the antagonistic substance, the negative effect of $MnSO_4$ being of higher magnitude than that of K_2HPO_4 [−0.24 and −0.10 log (AU mL^{-1}) units, respectively].

The only statistically significant effect of interaction was observed between $MnSO_4$ and sodium acetate (positive interaction) (Table 2). Figure 4 represents the variation of the mean values of bacteriocin titres obtained from the combinations of sodium acetate and $MnSO_4$, in the two BCM assayed. The corresponding response surface plot is shown in Fig. 3. The highest bacteriocin levels were registered in the presence of sodium acetate and absence of Mn^{2+} [log (AU mL^{-1}) = 2.95 ± 0.13]. In contrast, these levels significantly decreased when there was Mn^{2+}, but not sodium acetate in the culture media [log (AU mL^{-1}) = 2.57 ± 0.26]. However, simultaneous addition of the two salts enabled a high bacteriocin level [log (AU mL^{-1}) = 2.84 ± 0.27], attenuating the inhibitory main effect produced by Mn^{2+}.

The effects of factors evaluated on the bacteriocin production rates (Table 3) were similar to those obtained when analyzing the maximum bacteriocin levels (Table 2). Thus, the use of LAPTg instead of CNS-MRS, the presence of $MgSO_4$, sodium acetate and the simultaneous addition of $MnSO_4$ and sodium acetate (interaction effect) to the culture media affected the bacteriocin production rates positively. However, $MnSO_4$ exerted a negative influence on this kinetic parameter.

In most of the salt combinations assayed in both, LAPTg and CNS-MRS, the maximum bacteriocin activity was detected during the exponential phase of bacterial growth (6 h of culture) (Figs 1 and 2). For all culture conditions, the bacteriocin activity in the stationary phase decreased only slightly or remained stable. On the other hand, when maximum bacteriocin concentration was referred to the biomass produced at that specific time point (Tables 4 and 5, supporting information), the lowest values were obtained in the presence of $MnSO_4$ in the two BCM assayed. As described above, $MnSO_4$ biomass production positively, but affects bacteriocin production negatively.

Through the response surface plot it is possible to determine the value of the factors for optimal biomass and bacteriocin production, which were also calculated by applying the Statistica 6.0 software. According to the predictive results obtained, the optimum composition of the medium to obtain maximum bacteriocin and biomass production was: LAPTg as BCM, 0.1% ammonium citrate, 0.5% sodium acetate, 0.01% $MgSO_4$ and

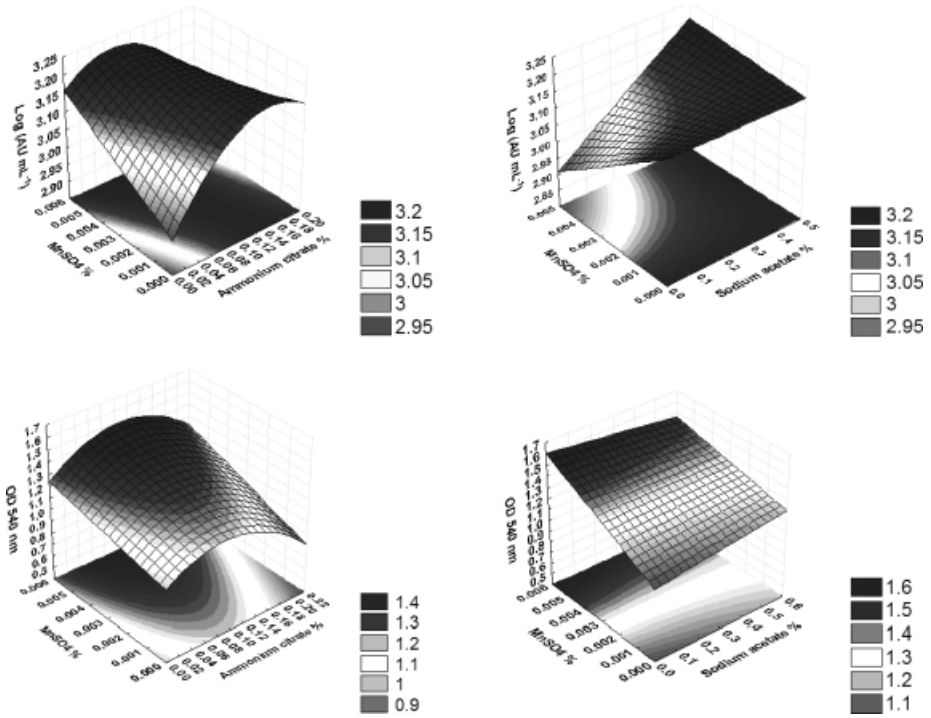

Figure 3. Response surface plot of bacteriocin production (upper panel) and growth cell (lower panel) showing the interaction effect between two salts (ammonium citrate – $MnSO_4$ and sodium acetate – $MnSO_4$) in LAPTg broth.

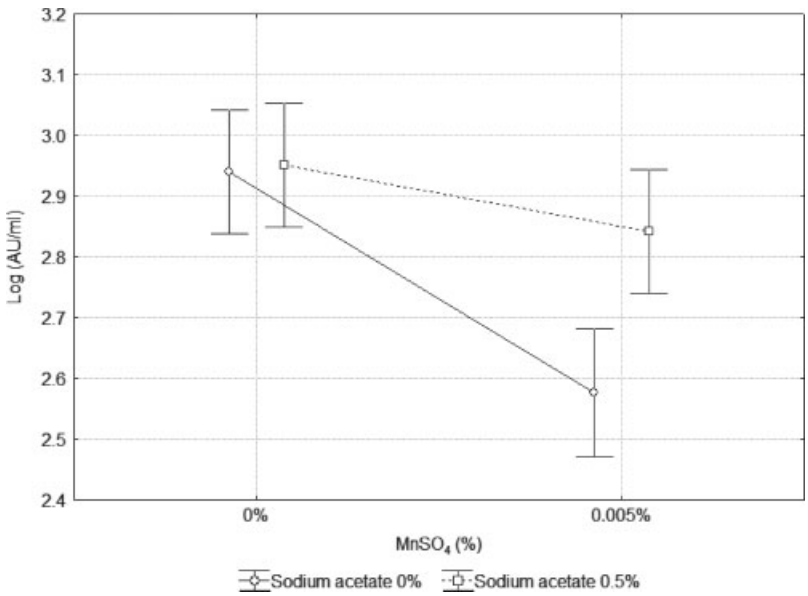

Figure 4. Bacteriocin levels produced by *L. salivarius* CRL 1328, according to the addition of sodium acetate and $MnSO_4$ to the culture media. Each value represents the mean and the 95% confidence interval of log (AU mL^{-1}) for each combination of sodium acetate and $MnSO_4$, in the two basal growth media assayed (LAPTg and CNS-MRS).

0.005% $MnSO_4$. A validation experiment was carried out using the optimized medium, and the maximum values obtained were: $OD_{540\ nm} = 1.34$ and log (AU mL^{-1}) = 3.16 (growth curves not showed).

DISCUSSION

Several culture media have been developed to support the robust growth of LAB, being also frequently employed to determine the production of different antagonistic substances, such as bacteriocins. For example, MRS medium was used to evaluate the factors affecting the production of plantaricin S and T of *Lactobacillus plantarum* LPCO10,[20] amylovorin L471 of *Lactobacillus amylovorus* DCE 471,[22] sakacin K of *Lactobacillus sakei* CTC 494,[4] pediocin PD-1 of *Pediococcus damnosus*,[23] etc. Audisio *et al.*[24] studied the effects of complex carbon sources on bacteriocin production by *Enterococcus faecium* CRL 1385 by employing LAPT broth. M17 medium, which was developed

Table 3. Influence of culture media composition on specific growth rates and bacteriocin production rates of *L. salivarius* CRL 1328

Factor	Kinetic parameters: Specific growth rates (h^{-1})	Kinetic parameters: Bacteriocin production rates (h^{-1})
Basal culture medium (BCM)[a]	NS	$p < 0.005$[b]
Ammonium citrate (AC)	NS	NS
Sodium acetate (SA)	NS	$p < 0.05$[b]
$MgSO_4$	NS	$p < 0.005$[b]
$MnSO_4$	NS	$p < 0.0005$[c]
K_2HPO_4	NS	NS
AC × SA	NS	NS
AC × $MgSO_4$	NS	NS
AC × $MnSO_4$	NS	NS
AC × K_2HPO_4	NS	$p < 0.05$[c]
SA × $MgSO_4$	NS	NS
SA × $MnSO_4$	NS	$p < 0.05$[b]
SA × K_2HPO_4	NS	NS
$MgSO_4$ × $MnSO_4$	NS	NS
$MgSO_4$ × K_2HPO_4	NS	NS
$MnSO_4$ × K_2HPO_4	NS	NS

[a] Basal culture media: LAPTg and CNS-MRS.
[b] Positive effects on bacteriocin production rates. For the BCM, it indicates higher bacteriocin production rates determined in LAPTg than in CNS-MRS; for the salts, it indicates higher bacteriocin production rates obtained when each salt is added to the growth medium.
[c] Negative effects on bacteriocin production rates
NS: Effect not significant. $P < 0.05$: Effect statistically significant.

to support the growth of lactococci and streptococci,[25] was employed to assay the levels of nisin production in several strains of *Lactococcus lactis* and enterocin 1146 production of *Enterococcus faecium* DPC1146.[26,27] Some of these experiments were performed by classical microbiological methodology.

Factorial designs and statistical analysis are valuable and efficient tools to study and optimize media constituents for growth and bacteriocin production, and have been applied for food-related bacteriocins, e.g. nisin.[6,7,19,20] To date, this procedure has not been applied for lactobacilli for pharmaceutical applications, or for a specific *L. salivarius* strain. The optimization of the medium ingredients for biomass and inhibitory substance production by vaginal *L. salivarius* CRL 1328 is one fundamental step towards the pilot and large-scale production of both responses, for their inclusion as bioactive principles in pharmaceutical probiotic products.

In this work, the statistical methodology applied was very useful to identify the main and interaction effects of different factors on biomass and bacteriocin production of *L. salivarius* CRL 1328. Among all the factors evaluated (five salts and basal culture medium), only the addition of Mn^{2+} to the growth media assayed produced a significant biomass increase of *L. salivarius* CRL 1328. These results demonstrate the requirement for this ion for the optimum cell growth of this specific strain. The essential requirement for Mn^{2+} by several *Lactobacillus* species has previously been reported.[5,28,29] For example, Leroy and De Vuyst[5] demonstrated that the omission of Mn^{2+} from MRS medium resulted in a substantial decrease in *L. sakei* CTC 494 growth.

The stimulatory effect of Mn^{2+} on the growth of LAB is associated with the activation of enzymes involved in the Krebs cycle, or in the homo- or heterofermentative pathways (e.g. glycolysis and pentose via, respectively) of carbohydrate metabolism in the same organism. Another effect of Mn^{2+} was detected through the protection of microbial cells from oxygen toxicity through substitution of superoxide dismutase in scavenging the superoxide radical, and stabilization of subcellular entities such as the native conformation of ribosomes, bacterial membranes and cell wall.[28,29]

On the other hand, the influence of carbon and nitrogen sources and organic and inorganic salts on bacteriocin production could be strain specific.[30] The nitrogen source has a major influence on the growth and bacteriocin synthesis, as reported in several studies.[4,27,31] In previous work, we reported that the favorable effects of nitrogen source (mainly yeast extract and tryptone) on biomass and bacteriocin production of *L. salivarius* CRL 1328 were higher than those of the carbon source (glucose and lactose).[32] Therefore, the higher bacteriocin production obtained in LAPTg when compared with CNS-MRS could be attributed to the higher amount of total nitrogen sources present in the first medium. In a similar way, Aktypis *et al*.[31] demonstrated that the increase of complex nitrogen sources concentration in the culture medium affected positively the synthesis of thermophilin T of *Streptococcus thermophilus* ACA-DC 0040. The authors suggested that the increase of peptides and/or growth factors derived from nitrogen sources contributes to additional bacteriocin production, since they are essential or could act as inducers for its synthesis.

In the present paper, the different inorganic sulfates assayed have different effects on the bacteriocin production of *L. salivarius* CRL 1328: $MgSO_4$ stimulated bacteriocin production, while $MnSO_4$ drastically limited salivaricin formation. These results suggest that manganese and magnesium ions, but not sulfate, play an important role in the metabolic pathways of this microorganism, in the range of concentrations assayed in the experimental design. Different to our results, Mn^{2+} but not Mg^{2+} improved pediocin PD-1 production and bacteriocin production of *L. sakei* CTC 494.[5,23] However, pediocin AcH levels have been shown to increase in the presence of both Mg^{2+} and Mn^{2+}.[10,33] Meghrous *et al*.[34] demonstrated that Mg^{2+} exerts a stimulatory effect on nisin production and decreases bacteriocin adhered to bacterial cells of *Lc. lactis subsp. lactis* ATCC 11 454.

The strong inhibition of salivaricin CRL 1328 production exerted by the presence of Mn^{2+} in culture media could be explained by the fact that the microbial cells, which are living in an optimum environment for their growth, reduce the synthesis of defensive molecules and employ the available nutrients mainly for biomass production.[4] However, when Mn^{2+} and sodium acetate were simultaneously added to the culture media, the bacteriocin production increased to values comparable with the optimal reached in LAPTg. Thus, the relevant positive interaction effect between $MnSO_4$ and sodium acetate on the bacteriocin levels enabled the optimal combination and concentration of ingredients in a culture medium that stimulated both bacteriocin and biomass production. Biomass and bacteriocin production may be affected by initial and final pH as well as by pH drop in the cultures (due to lactic acid production).[35] In previous studies, we demonstrated that the decrease in the pH of the growth medium was parallel to the growth of *L. salivarius* CRL 1328.[21] The differences between initial and final pH and the pH decrease rate were dependent on the initial pH of the broth in both LAPTg and MRS media, as was also demonstrated in that work. In the same way, the initial pH of the culture medium had a dramatic effect on the production of bacteriocin. Therefore, in this work, the initial

pH of the different culture media assayed was adjusted to the optimal value for growth and bacteriocin synthesis, supported by the previous results.

The increased bacteriocin levels in the presence of sodium acetate could be due to a pH effect, because of the buffering capability of the added acetate. In a similar way, the addition of sodium acetate to the culture medium, both individually and combined with sodium citrate and KH_2PO_4, produced a beneficial effect on leuconocin Lcm1 and sakacina A synthesis, which suggests that the production of these bacteriocin requires a high pH.[36] However, the lower pediocin AcH production in buffered media (containing sodium citrate, sodium acetate and KH_2PO_4) could be due to the requirement of a low pH (<5.0) for the efficient processing of prepediocin molecule to active pediocin AcH.[10]

On the other hand, phosphate control is a well known regulatory mechanism of the production of inhibitory substances.[37,38] Inorganic phosphates exerted a beneficial effect on nisin production of several *Lactococcus lactis* strains.[6,7,37] The negative influence of K_2HPO_4 on salivaricin CRL 1328 levels, in the range evaluated in this study, could be explained by the inhibition of production, release, or on the action of different molecules involved in the inhibitory substance synthesis (synthetases, inducers, precursor, etc.).[37,38] However, further studies are required to elucidate this hypothesis.

The maximum activity of salivaricin CRL 1328 was always observed during the exponential growth phase, while there was not a significant decrease of bacteriocin activity in the stationary phase. These results indicate that the production of salivaricin displays a primary metabolite kinetic, as previously reported.[12,21] Similarly, thermophilin T is a primary metabolite, since its synthesis is a growth-associated process.[31] However, thermophilin T activity progressively decreased during the stationary phase, which seemed to be related with glucose depletion in the growth medium.

On the other hand, Vázquez and Murado[35] described that nisin and pediocin production of *Lactococcus lactis* HD1 and *Pediococcus acidilactici* NRRL B-5627, respectively, follows a similar kinetic profile to that of the cell growth. However, through the application of a mathematical model to describe the kinetics of growth, bacteriocins and lactic acid production of both microorganisms, the authors determined that the rate of formation of bacteriocin depended on the biomass production rate and on the biomass present in the culture medium. Therefore, a mixed metabolite kinetic of nisin and pediocin formation was described in that system.

In conclusion, $MnSO_4$ was indispensable for the optimum growth of *L. salivarius* CRL 1328, but produced a negative effect on bacteriocin synthesis. However, the positive effects of sodium acetate and $MgSO_4$ allowed increasing bacteriocin levels, even in the presence of Mn^{2+}. The results obtained are potentially applicable to the technological production of probiotic bacteria and antagonistic substances. Further studies are being performed in order to combine some low cost carbon and nitrogen sources with different salts to design a more economical culture medium than standard media, for the production of biomass and bacteriocin of *L. salivarius* CRL 1328.

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Supporting information

Supporting information may be found in the online version of this article.

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